Inflammasome Activity in the Inflammatory Bowel Diseases

Nicole Maree Ranson

B. Sc. 1989

BBiomedSc (Hons) 2013

Submitted in fulfilment of the requirements

for the degree of Doctor of Philosophy

University of Tasmania

May 2018

DECLARATION OF ORIGINALITY

This thesis entitled "Inflammasome Activity in the Inflammatory Bowel Diseases" contains original research conducted by the candidate within the School of Health Sciences at the University of Tasmania, and contains no material which has been accepted for a degree or diploma by the University or any other institution, except by way of background information and duly acknowledged in the thesis, and to the best of my knowledge and belief no material has previously been published or written by another person except where due acknowledgement is made in the text of the thesis, nor does the thesis contain any material that infringes copyright.

_____

Nicole M. Ranson May 2018

AUTHORITY OF ACCESS

This thesis is not to be made available for loan or copying for two years following the date this statement was signed. Following that time the thesis may be made available for loan and limited copying in accordance with the *Copyright Act 1968*.

_____

Nicole M. Ranson May 2018

STATEMENT OF ETHICAL CONDUCT

The research associated with this thesis abides by the international and Australian codes on human and animal experimentation, the guidelines by the Australian Government's Office of the Gene Technology Regulator and the rulings of the Safety, Ethics and Institutional Biosafety Committees of the University.

_____

Nicole M. Ranson May 2018

STATEMENT REGARDING PUBLISHED WORK

The publishers of the papers comprising Chapters 1, 3, 5 and 6 hold the copyright for that content, and access to the material should be sought from the respective journals. The remaining non published content of the thesis may be made available for loan and limited copying and communication in accordance with the above Statement of Access and the *Copyright Act 1968*.

_____

Nicole M. Ranson May 2018

LIST OF PUBLICATIONS AND STATEMENT OF CO-AUTHORSHIP

The following people and institutions contributed to the publication of work undertaken as part of this thesis.

Candidate: Nicole M Ranson[1]

Other authors: Rajaraman D. Eri[1] (RDE)

Anthony L. Cook[4] (ALC)

Dale Kunde[1] (DK)

Mark Veldhuis[2,3] (MV)

Brent Mitchell[2,3] (BM)

Scott Fanning[2,3] (SF)

[1] School of Health Sciences, University of Tasmania, Launceston, Tasmania, 7250, Australia

[2] Launceston General Hospital, Launceston, Tasmania, 7250, Australia

[3] St Vincent's Hospital, Calvary Health Care, Launceston, Tasmania, 7250, Australia

[4] Wicking Dementia Research and Education Centre, Faculty of Health, University of Tasmania, Hobart, Tasmania, 7000, Australia

Author details and their roles:

Paper 1:

Ranson N, Kunde D, Eri R, Regulation and Sensing of Inflammasomes and Their Impact on Intestinal Health, *International Journal of Molecular Science*. 2017;18(11): 2379,

doi: 10.3390/ijms18112379.

Located in Chapter 1

Candidate was the primary author. RDE and DK critically reviewed the manuscript.

Paper 2:

Ranson N, Veldhuis M, Mitchell B, Fanning S, Cook AL, Kunde D, Eri R, Nod-Like Receptor Pyrin-Containing Protein 6 (NLRP6) is Upregulated in Ileal Crohn's Disease and Differentially Expressed in Goblet Cells, *Cellular and Molecular Gastroenterology and Hepatology,* 2018, doi: 10.1016/j.jcmgh.2018.03.001

Located in Chapters 3 and 6

Candidate performed laboratory analyses and wrote the manuscript. RDE, ALC and DK supervised the development of work. DK analysed the RNA sequencing data. MV,BM, SF collected biopsy samples. All authors critically reviewed the manuscript before submission.

Paper 3:

Ranson N, Veldhuis M, Mitchell B, Fanning S, Cook AL, Kunde D, Eri R, NLRP3 Dependent and Independent Processing of Interleukin (IL)-1β in Active Ulcerative Colitis, *Inflammatory Bowel Disease* (under review)

Located in Chapters 3 and 5

Candidate performed laboratory analyses and wrote the manuscript. RDE, ALC and DK supervised the development of work. MV,BM, SF collected biopsy samples. All authors critically reviewed the manuscript before submission.

Paper 4:

De Santis S, Kunde D, Serino G, Galleggiante V, Caruso ML, Mastronardi M, Cavalcanti E, **Ranson N**, Pinto A, Campiglia P, Santino A, Eri R, Chieppa M, Secretory Leukoprotease Inhibitor is Required for Efficient Quercetin-Mediated Suppression of TNFα Secretion, *Oncotarget*, 2016: 7(46): 75800-75809, doi: 10.18632/oncotarget.12415

Additional Publication: work not included in the thesis.

Candidate performed immunohistochemistry analyses and assisted with manuscript writing and review.

We, the undersigned agree with the above stated "proportion of work undertaken" for each of the above published (or submitted) peer-reviewed manuscripts contributing to this thesis:

Rajaraman D. Eri	Anthony L. Cook
Dale Kunde	Mark Veldhuis
Brent Mitchell	Scott Fanning

CONFERENCE POSTER PRESENTATIONS

Hierarchical Inflammasome Activation in the Inflammatory Bowel Diseases

17th International Congress of Mucosal Immunology (ICMI 2015), 14th - 18th July 2015, Berlin, Germany.

Inflammasomes and Intestinal Inflammation

International Congress of Immunology (ICI 2016), 21st – 26th August 2016, Melbourne, Australia.

The NLRP3 Inflammasome is Differentially Activated During Active Ulcerative Colitis and Crohn's Disease

18th International Congress of Mucosal Immunology (ICMI 2017), 19th – 22nd July 2017, Washington D.C., United States of America.

ACKNOWLEDGEMENTS

I would like to thank the School of Health Sciences for giving me the opportunity to study within the Faculty and the Clifford Craig Medical Research Trust for providing funding support for this project.

My studies have been supported by so many people and in particular I would like to extend my sincere gratitude to:

Dr Rajaraman Eri, my primary supervisor, for his direction and boundless optimism.

Dr Anthony Cook, my co-supervisor, for always providing support and exceptional feedback.

Dr Dale Kunde, my co-supervisor, for his technical support, guidance and encouragement throughout my candidature.

Royal Hobart Hospital and Tony Van Galen, for their generous donation of a Dako Cytomation Pascal Pressure Chamber which was used for immunohistochemistry and immunofluorescence confocal microscopy analyses.

And finally, to my friends, Waheedha Basheer and Sarron Randall-Demllo your conversations and laughs in the laboratory will be missed.

THESIS SUMMARY

The work contained in this thesis would not have been possible without the collaboration from local clinicians and support from local private enterprises within the Launceston community. During the course of this PhD, immunohistochemistry and immunofluorescence confocal microscopy techniques were developed which are now routinely used by the Mucosal Immunology team, located within the School of Health Sciences at UTAS, Newnham.

A key initiating event in the development of Inflammatory Bowel Diseases (IBD) is thought to be activation of the inflammasome complex and therefore the aim of this study was to examine the mRNA expression of inflammasome component and their cellular localisation in remission and active IBD. The structure of the thesis includes a General Introduction, a Material and Methods Chapter, followed by three Research Chapters and a General Discussion. With this thesis design there is however some repetition, particularly in the introduction sections, due to the similarity of background material.

In summary, Chapter 3 of this thesis details the mRNA expression of targeted inflammasome genes in biopsies obtained from ulcerative colitis (UC) and Crohn's disease (CD) patients. The most exciting finding related to this work was the disease specific upregulation of *NLRP6* in ileal CD, which was confirmed by both qRT-PCR performed at UTAS and RNA sequencing performed independently at Ramaciotti Centre for Genomics in Sydney.

Chapter 4 reports research focused on the AIM2 inflammasome. From Chapter 3 it was established that the expression of *AIM2* increased with disease activity in

UC and CD. Using immunohistochemistry and immunofluorescence confocal microscopy AIM2 was localised to epithelial cell layer, specifically to the intraepithelial lymphocytes, which are cells at the forefront of immune defences. Chapter 5 details the cellular localisation and spatial relationship of NLRP3 and Interleukin (IL)-1β and follows on from the upregulation of *NLRP3* and *IL-1β* in active disease, demonstrated in Chapter 3. In general, NLRP3 was found to be predominantly expressed in neutrophils and other immune cells of the lamina propria. NLRP3 is generally regarded as a global sensor because of its ability to process IL-1β in response to a wide range of pathogenic, sterile and cell stress activators. The product of inflammasome activation, IL-1β is a potent inflammatory cytokine responsible for many local and systemic responses. In the normal colon, inflammasome dependent caspase-1 was found to contribute to the production of mature IL-1β. Surprisingly and novel to this research was the reduced contribution of the NLRP3 inflammasome to IL-1β production in active UC. Given the dominance of a neutrophil lamina propria cell population in active UC it was likely that neutrophil derive serine proteases contributed more than the NLRP3 inflammasome to the overall IL-1β production.

In the murine system, NLRP6 has been shown to regulate goblet cell mucin production and secretion, regulate epithelial self-renewal and proliferation, protect against chemical induced intestinal injury and tumorigenesis, and negatively regulate inflammasome signalling. In contrast, human studies examining the expression and activity of NLRP6 remain limited.

Chapter 6 details research carried on from the disease specific upregulation of *NLRP6* in ileal CD and describes the colonic localisation of NLRP6 and MUC2 in active and remission IBD. In ileal CD, NLRP6 was localised to the epithelial cell

layer, myofibroblasts and lamina propria immune cells. Consistent with clinical observation MUC2 was increased in CD and reduced in active UC. The identification of a high NLRP6 expressing goblet cell localised to the apical region of the intestinal crypt was a novel and exciting finding. Furthermore, the effect of NLRP6 induction on MUC2 expression in human colonic cell lines was a new avenue of investigation and demonstrated the repression of *MUC2* expression with *NLRP6* induction. The relationship of NLRP6 to MUC2 and E-cadherin was intriguing and will be the focus of future research.

In conclusion, this research project has described the colonic localisation of AIM2, NLRP3, IL-1β, MUC2 and NLRP6 in the normal colon, remission and active UC and CD. Novel findings include;

1. The disease specific upregulation of *NLRP6* in active ileal CD.
2. The identification of a NLRP6 expressing goblet cell localised predominantly in the upper portion of the intestinal crypt, which suggests a possible role for NLRP6 in goblet cell expulsion.
3. The intraepithelial lymphocyte localisation of AIM2 in normal, remission and active disease which suggests AIM2 is geared to provide immediate and heightened immune protection.
4. The reduced contribution of NLRP3 to IL-1β in active UC suggests neutrophil- derived serine proteases are likely the source of bioactive IL-1β and caspase-1 has only a minor role.

Altogether, this study has provided an insight into the activity of inflammasome in human IBD. NLRP6 can now be considered a potential marker for distinguishing ileal CD from colonic CD and terminal ileum involved UC. Future work needs to address if there is a therapeutic benefit in switching on NLRP6 in

active UC or if disabling NLRP6 improves disease activity in ileal CD. Similarly, is there a therapeutic benefit in blocking both caspase-1 and neutrophil derived serine proteases in active UC?

THESIS ABSTRACT

Ulcerative colitis (UC) and Crohn's disease (CD) are characterised by chronic and recurrent inflammation of the gastrointestinal tract. The development of Inflammatory Bowel Diseases (IBD) is regarded as a multifactorial disease process involving a combination of immune system defects and environmental influences, which occur in the genetically predisposed individual. In the gut, the multimolecular complex known as the inflammasome is a key mediator in regulating the host's immune response to invading pathogens and/or cellular stress. Formation of the inflammasome complex provides a platform for the caspase-1 dependent activation of the potent inflammatory cytokines, interleukin (IL)-1β and IL-18. Dysregulation of the inflammasome complex is thought to contribute to IBD pathogenesis.

Quantitative RT-PCR, RNA-sequencing, immunohistochemistry and immunofluorescence confocal microscopy were used to examine the mRNA expression and cellular localisation of inflammasome forming components in colonic biopsies obtained from IBD and control patients. Rosiglitazone induced *NLRP6* expression was performed in the colonic cell lines, LS174T and HT29 to investigate the effect of *NLRP6* on *MUC2* expression.

Novel findings from this research include the disease specific upregulation of *NLRP6* in active ileal CD. The identification of a NLRP6 expressing goblet cell localised predominantly in the upper portion of the intestinal crypt. Repression of *MUC2* expression with increased expression of *NLRP6* in the human colorectal adenocarcinoma cell line, LS174T. The localisation of AIM2 to the intraepithelial

lymphocytes and the reduced contribution of NLRP3-dependent caspase-1 to bioactive IL-1β production in active UC.

In conclusion, NLRP6 can now be considered a potential marker for distinguishing ileal CD from colonic CD and terminal ileum involved UC. Furthermore, results of this study will direct future work examining if there is a therapeutic benefit in switching off NLRP6 in active ileal CD, or blocking both caspase-1 and neutrophil-derived serine protease production of IL-1β in active UC.

TABLE OF CONTENTS

DECLARATION OF ORIGINALITY I

AUTHORITY OF ACCESS I

STATEMENT OF ETHICAL CONDUCT II

STATEMENT REGARDING PUBLISHED WORK II

LIST OF PUBLICATIONS AND STATEMENT OF CO-AUTHORSHIP III

CONFERENCE POSTER PRESENTATIONS VII

ACKNOWLEDGEMENTS VIII

THESIS SUMMARY IX

THESIS ABSTRACT XIII

TABLE OF CONTENTS XV

LIST OF FIGURES XIX

LIST OF TABLES XXIII

LIST OF ABBREVIATIONS XXIV

CHAPTER 1 GENERAL INTRODUCTION 1

1.1 INTRODUCTION 1

1.2 INFLAMMATORY BOWEL DISEASES 4

1.3 GENETIC RISK FACTORS 7

1.4 ENVIRONMENTAL RISK FACTORS 9

1.5 THE INNATE IMMUNE SYSTEM 10

1.5.1 Intestinal Epithelial Barrier 10

1.5.2 Pathogen Receptors of the Innate Immune System 11

1.6 THE INFLAMMASOME COMPLEX 13

1.6.1 Formation of a NOD-Like Receptor Protein (NLRP) Inflammasome Complex 14

1.6.2 Structure and Formation of a Pyrin and HIN Domain (PYHIN) Inflammasome Complex 20

1.7 LIGAND SENSING OF INFLAMMASOME COMPLEXES 22

1.7.1 The NLRP1 Inflammasome 22

1.7.2 The NLRP3 Inflammasome 23

1.7.3 The NLRC4 Inflammasome 27
1.7.4 The AIM2 and IFI16 Inflammasome 29
1.7.5 The NLRP6, NLRP7 and NLRP12 Inflammasomes 30
1.8 Regulation of the inflammasome Complex 31
1.8.1 Regulation by Autoinhibition of the Ligand Sensing Domain 32
1.8.2 Priming Events that Regulate Activation 32
1.8.3 Regulation by POPs and COPs 33
1.8.4 Regulation by Type I Interferons 34
1.8.5 Regulation from Inflammasome Components 35
1.9 Disease Associated Inflammasome Related Variants 36
1.10 Inflammasomes and their Impact on the Intestinal Environment 41
1.11 Aims and Hypothesis 43
CHAPTER 2 MATERIAL AND METHODS 44
2.1 Ethics 44
2.2 Study Participants and Biopsy Collection 44
2.3 RNA Isolation 45
2.4 Transcriptional Profiling with RNA-Sequencing 46
2.5 complementary DNA Preparation 47
2.6 Gene Expression Analysis 47
2.7 Immunohistochemistry Analysis 49
2.8 Immunofluorescence Confocal Microscopy 50
2.9 Cell Culture Experiments 53
2.10 Data Analysis 55
CHAPTER 3 GENE EXPRESSION OF INFLAMMASOME COMPONENTS IN THE INFLAMMATORY BOWEL DISEASES 58
3.1 Introduction 58
3.2 Results 62
3.2.1 Study Participant Demographics 62
3.2.2 Clinical Characteristics of Study Participants 62
3.2.3 Expression of Targeted Inflammasome Genes in IBD 67
3.2.4 Correlation of Inflammasome Component mRNA in Active Disease 68
3.3 Discussion 77

CHAPTER 4 COLONIC LOCALISATION AND INTRAEPITHELIAL LYMPHOCYTE EXPRESSION OF AIM2 80

4.1 INTRODUCTION 80

4.2 RESULTS 83

4.2.1 AIM2 Antibody Optimisation 83

4.2.2 Colonic Localisation of AIM2 85

4.3 DISCUSSION 90

CHAPTER 5 COLONIC LOCALISATION AND SPATIAL INTENSITY RELATIONSHIP OF NLRP3 AND INTERLEUKIN (IL)-1BETA 92

5.1 INTRODUCTION 92

5.2 RESULTS 95

5.2.1 NLRP3 and IL-1β Antibody Optimisation 95

5.2.2 Colonic Localisation of NLRP3 99

5.2.3 Colonic Localisation of IL-1β 104

5.2.4 Colocalisation of NLRP3 and IL-1β in Active UC and CD 110

5.3 DISCUSSION 115

CHAPTER 6 COLONIC LOCALISATION OF NLRP6 AND INTERACTION WITH MUC2 AND E-CADHERIN 119

6.1 INTRODUCTION 119

6.2 RESULTS 122

6.2.1 NLRP6 Antibody Optimisation 122

6.2.2 Colonic Localisation of NLRP6 124

6.2.3 MUC2 Antibody Efficiency and Colonic Localisation 128

6.2.4 Colocalisation of NLRP6 with MUC2 and E-cadherin 134

6.2.5 The Induction of NLRP6 in Colonic Cell Lines 137

6.3 DISCUSSION 140

CHAPTER 7 GENERAL DISCUSSION 143

7.1 DISCUSSION 143

7.2 LIMITATION OF THIS STUDY 150

7.3 FUTURE DIRECTION 152

APPENDICES 154

APPENDIX 1: CALVARY HEALTH CARE APPROVAL LETTER 154

APPENDIX 2: UNIVERSITY OF TASMANIA ETHICS APPROVAL 155

APPENDIX 3: PATIENT PARTICIPATION INFORMATION FOR THE IBD STUDY 157

APPENDIX 4: MEDICAL HISTORY AND CONSENT FORM FOR IBD STUDY 160

APPENDIX 5: SELECTION OF SUITABLE HOUSEKEEPING GENE FOR QRT-PCR NORMALISATION 162

APPENDIX 6: PRODUCT INFORMATION SHEET FOR THE AIM2 ANTIBODY (AB93015, ABCAM, CAMBRIDGE, MA) 163

APPENDIX 7: PRODUCT INFORMATION SHEET FOR THE NLRP6 ANTIBODY (NBP2-31372, NOVUS BIOLOGICAL, LITTLETON, CO, USA) 165

REFERENCES 168

LIST OF FIGURES

Figure 1-1: The Structure of the colonic mucosa within the human gut……3

Figure 1-2: Structure of the human NOD-like receptor subgroups..... 16

Figure 1-3: Formation of a NOD-like receptor protein inflammasome containing an N-terminal pyrin domain..... 18

Figure 1-4: The mechanism for the inflammasome mediated catalytic conversion of procaspase-1 to caspase-1 19

Figure 1-5: Structure of the human pyrin and HIN domain (PYHIN) family..... 21

Figure 3-1: Gene expression of inflammasome related genes in paired biopsies from quiescent CD (qCD), active CD (aCD), quiescent UC (qUC) and active UC (aUC)..... 72

Figure 3-2: Targeted RNA-sequencing analysis of ileal CD colon biopsies during active disease..... 73

Figure 3-3: Targeted RNA-sequencing analysis of colonic CD colon biopsies during active disease. 74

Figure 3-4: Correlation of *NLRP3* to *IL-1β* in active UC and active CD 76

Figure 4-1: Localisation of AIM2 in normal human tonsil tissue as analysed by immunohistochemistry. 84

Figure 4-2: Representative immunofluorescence confocal images of AIM2 localisation in normal human tonsil tissue 84

Figure 4-3: Representative immunohistochemistry images of AIM2 expression in normal colon, active UC, remission UC, active ileal and colonic CD and remission CD. 87

Figure 4-4: Representative immunofluorescence confocal images of AIM2 localisation in normal colon, active UC, active ileal and colonic CD..... 87
Figure 4-5: Quantification of AIM2 immunohistochemistry staining in biopsy sections from normal and IBD patients..... 89
Figure 5-1: Representative immunohistochemistry images of NLRP3 and IL-1β localisation in human control material..... 96
Figure 5-2: Representative immunofluorescence confocal images of NLRP3 and IL-1β localisation in human control material..... 98
Figure 5-3: Representative immunohistochemistry images of NLRP3 expression in normal colon, active and remission UC, active ileal and colonic CD and remission CD..... 102
Figure 5-4: Representative immunofluorescence confocal images of NLRP3 expression in normal colon, active UC and active ileal and colonic CD..... 102
Figure 5-5: Representative immunohistochemistry images of IL-1β expression in normal colon, active UC, remission UC, active ileal and colonic CD and remission CD..... 107
Figure 5-6: Representative immunofluorescence confocal images of IL-1β expression in normal colon, active UC and active ileal and colonic CD..... 107
Figure 5-7: Quantification of NLRP3 and IL-1β immunohistochemistry staining in biopsy sections from normal and IBD patients..... 109
Figure 5-8: Representative co-immunostaining images of NLRP3 (red) and IL-1b (green) in normal colon and active UC and active CD..... 111
Figure 5-9: Manders colocalisation coefficients (M1 and M2) for **A)** normal colon, **B)** active UC, **C)** active colonic CD, **D)** active ileal CD..... 113

Figure 5-10: Pearson's correlation coefficients for normal colon, active UC and active CD. 114

Figure 6.1: Localisation of NLRP6 in normal human breast tissue as analysed by immunohistochemistry. 123

Figure 6-2: Representative immunofluorescence confocal images of NLRP6 localisation in normal human breast tissue 123

Figure 6-3: Representative immunohistochemistry images of NLRP6 expression in normal colon, active UC, remission UC, active ileal and colonic CD and remission CD. 126

Figure 6-4**:** Representative immunofluorescence images of NLRP6 localisation in normal colon, active UC, active ileal and colonic CD. 126

Figure 6-5: Representative immunohistochemistry images of MUC2 expression in normal colon, active UC, remission UC, active ileal and colonic CD and remission CD. 130

Figure 6-6: Representative immunofluorescence images of MUC2 localisation in normal colon, active UC, active ileal and colonic CD. 130

Figure 6-7: Quantification of NLRP6 and MUC2 immunohistochemistry staining in biopsy sections from normal and IBD patients 132

Figure 6-8: Representative colocalisation images of NLRP6, MUC2 and E-cadherin in normal colon biopsies. 133

Figure 6-9: Representative colocalisation images of NLRP6, MUC2 and E-cadherin in ileal CD biopsies. 136

Figure 6-10: mRNA expression of *NLRP6, MUC2, PPAR-γ, IL-1β* and *IL-18* in LS174T cells treated with Rosiglitazone. 138

Figure 6-11: mRNA expression of *NLRP6, MUC2, PPAR-γ, IL-1β* and *IL-18* in HT29 cells treated with Rosiglitazone. 139
Figure 7-1: Production of IL-1β in the normal colon and active UC..... 148
Figure 7-2: Activity of NLRP6 in ileal CD..... 149
Figure A6-1: Mean absolute Ct values for the four housekeeping genes across the 5 disease categories..... 162

LIST OF TABLES

Table 2-1: Pre-designed Taqman primer/probes used for gene expression analysis 48

Table 2-2: Primary antibodies used for immunofluorescence confocal microscopy 52

Table 3-1: Demographic details of study participants 64

Table 3-2: Clinical characteristics of ulcerative colitis patients 65

Table 3-3: Clinical characteristics of Crohn's disease patients 66

Table 3-4: Expression of targeted inflammasome related genes in IBD 69

Table 3-5: Correlation of inflammasome component mRNA in active UC and active CD 75

Table 7-1: Descriptive statistics for absolute Ct values of the four housekeeping genes 162

LIST OF ABBREVIATIONS

The following abbreviations are used in this thesis:

^{0}C	Degrees Celsius
AIM2	Absent in melanoma 2
ASC	Apoptosis-associated speck-like protein containing a CARD
ATP	Adenosine triphosphate
CASP1	Caspase-1
CD	Crohn's disease
CLR	C-type lectin receptor
cDNA	Complementary deoxyribonucleic acid
DAMPs	Damage-associated molecular patterns
dsDNA	Double stranded deoxyribonucleic acid
DSS	Dextran sodium sulphate
GWAS	Genome-wide association studies
IBD	Inflammatory bowel disease
IFN	Interferon
IL	Interleukin
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
MDP	Muramyl dipeptide
mROS	Mitochondrial reactive oxygen species
mRNA	Messenger ribonucleic acid
MUC2	Mucin 2

NLRP	Nod-like receptor pyrin containing protein
NOD1	Nucleotide-binding oligomerisation domain containing 1
NOD2	Nucleotide-binding oligomerisation domain containing 2
PAMPs	Pathogen-associated molecular patterns
PPAR-γ	Peroxisome proliferator-activated receptor gamma
qRT-PCR	Quantitative real-time polymerase chain-reaction
RNA	Ribonucleic acid
ROI	Regions of interest
ROS	Reactive oxygen species
SenGC	Sentinel goblet cell
SNP	Single nucleotide polymorphism
TLR	Toll-like receptor
TNF	Tumor necrosis factor
UC	Ulcerative colitis

CHAPTER 1 GENERAL INTRODUCTION

1.1 INTRODUCTION

The human gut is a 24 hours a day, 365 days a year continuous system that is responsible for converting the food we eat into the energy our bodies need to grow and develop. It helps with the removal of waste material by forming stools and plays an important role in protecting us from infection by fighting harmful bacteria, viruses and cancer cells. The gut is home to millions of microorganisms, many of which aid intestinal function by stimulating the digestive process and the absorption of nutrients [1].

The luminal contents of the gut are bound by three distinct cellular layers, the mucosa, submucosa and the muscularis propria. The lamina propria extends from the subepithelial basement membrane complex to the muscularis propria and is composed of various types of leucocytes, extracellular matrix and fibroblasts [2] (Figure 1-1).

At the mucosa surface numerous pathogenic microbes and environmental toxins challenge the vast array of immune cells within the lamina propria to direct and shape the body's immune system. The immune system consists of two equally important aspects, the innate immunity and the adaptive immunity. Both are important for maintaining defences but differ with respect to response time, central cell types and specificity for different classes of microbes.

The components of the innate immune system include the physical epithelial barriers that prevent infection, the leucocytes, proteins and mechanisms that are always present and ready to fight infection and the cytokines and chemokines, which recruit phagocytic cells to the site of infection. These responses are quickly

activated and are dependent on the recognition of pathogenic insult or cellular stress [3].

In contrast, the adaptive immune system lacks the ability to respond to infection immediately but acts on pathogens that have evaded and overcome the innate immune responses. There are two types of adaptive immune responses, humoral immunity, mediated by antibodies produced by B lymphocytes and cell-mediated immunity, mediated by T lymphocytes. Both cause proliferation of effector cells and provide a potent mechanism for neutralising and eliminating pathogens. Adaptive immunity creates immunological memory and confers long lasting protection for the body against the reinfection of specific pathogens [4].

The main challenge of the gut immune system is to remain unresponsive to the microorganisms that participate in the digestive process while regulating a dynamic microbial population that is easily influenced by lifestyle factors such as diet, medication, age and illness. When dysbiosis of the resident microbial population occurs, infection and malabsorption can often follow, and this can be a contributing factor in the development of gastrointestinal disease.

Diseases characterised by chronic and relapsing inflammation of the gastrointestinal tract are known as inflammatory bowel diseases (IBD). The two main types of IBD are ulcerative colitis (UC) and Crohn's disease (CD). The mechanisms that lead to the development of these diseases is still unclear, however it is generally regarded as a multifactorial disease process involving a combination of immune system defects and environmental influences which occur in the genetically predisposed individual [2].

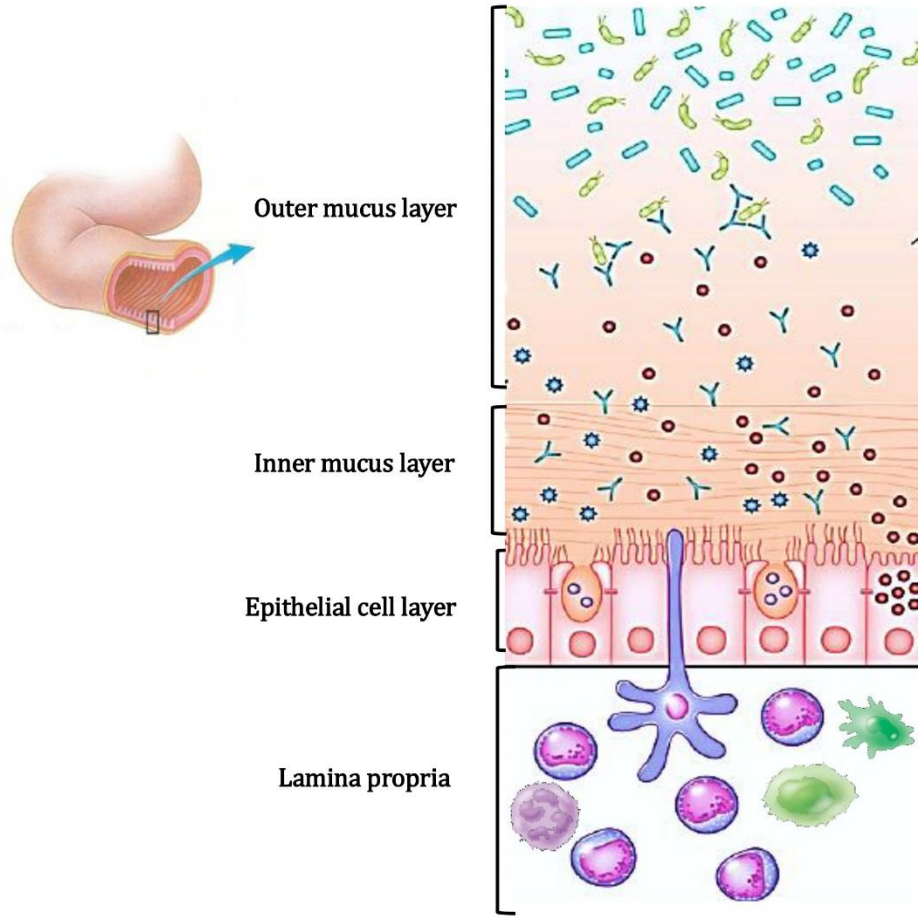


Figure 1-1: The Structure of the colonic mucosa within the human gut.

The epithelial cell layer of the gut separates the contents of the gut lumen from the immune cells of the lamina propria and is comprised of absorptive enterocytes, goblet cells, Paneth cells and enteroendocrine cells. Goblet cells contribute to the protective mucus layer and promote the elimination of gut contents [5]. The lamina propria immune cells, such as dendritic cells, neutrophils, monocytes, lymphocytes and macrophages either detect pathogens or are recruited to the site of infection or injury to initiate downstream innate and adaptive immune system pathways that promote pathogen elimination and tissue repair [2] (image adapted from Kim, Y and Ho, S [5]).

1.2 INFLAMMATORY BOWEL DISEASES

In Australia, it is estimated that IBD affects 1 in 250 people aged 5-49 and that by the year 2020 more than 92,000 people will be diagnosed with the disease. In 2012, the management of IBD was estimated to cost Australia, $270 billion with a loss of productivity at around $380 million [6]. Australia along with other western, industrialised countries such as United States, Canada, New Zealand and certain European Countries have the highest prevalence of IBD while Asian countries report the lowest incidence and prevalence [7-9].

The symptoms of IBD can vary, but in general patients usually present with one or more of the following; watery or bloody diarrhoea, rectal bleeding, faecal incontinence, abdominal pain, tenesmus (pain when passing stools), faecal urgency, fever, weight loss, malnutrition and lethargy [10-13]. In addition, more than 30% of IBD patient experience other chronic inflammatory disease such as primary sclerosing cholangitis, ankylosing spondylitis, psoriasis and arthritis [2, 10].

In the absence of infectious causes, a diagnosis of UC or CD involves the combination of clinical, laboratory, radiographic and endoscopic observations. Multiple endoscopic mucosal biopsies are useful for assessing disease activity and the identification of pre-cancerous lesions in UC, however in CD they tend not to be as useful because CD lesions frequently occur adjacent to normal mucosa [11, 14].

UC primarily affects the mucosa and submucosa layers of the large intestine. Usually the disease begins at the anal verge and spreads proximally in a diffuse and continuous manner along the length of the colon. In Pancolitis the inflammation extents along the entire length of the colon to the ileocaecal valve

and occasionally involves the terminal ileum [11]. The gross appearance varies with disease activity but in general, there is evidence of mucosal erosion, mucin depletion, oedema, petechial haemorrhages and mucosal ulcers or lesions of varying size [15].

Microscopically, UC is characterised by structural abnormalities of the mucosa and increased intensity of lamina propria cellular infiltrates with alteration in composition and change in distribution. Often neutrophils within epithelial structures, such as crypt wall (cryptitis), crypt lumen (crypt abscess) or associated with crypt destruction indicates active disease. Other features such as paneth cell metaplasia, diffuse thickening of the muscularis mucosae may help confirm diagnosis [16].

In contrast, the inflammation in CD is discontinuous and transmural and can affect any area of the gastrointestinal tract, from mouth to anus. The most common sites are the terminal ileum, colon and ileo-caecal region, while involvement of the upper gastrointestinal tract is uncommon [11]. In CD, the length of involved disease is variable and lesions are separated by uninvolved 'skip areas'. The gross appearance of the mucosa is often heterogeneous with multiple aphthoid lesions. Over time these lesions confluent both transversely and longitudinally to give the mucosa the distinctive cobblestone appearance. Ulcerations can give rise to strictures, abscesses or deeply situated fissuring ulcers which reach adjacent organs, all of which are not normally seen in UC [10]. Preserved or increased mucin secretion is also a feature of CD [11].

Microscopic assessment of disease activity in CD is difficult because of the segmental and transmural nature of the disease and biopsies of early lesions (preaphthoid lesions) do not yield diagnostic information. Features that favour

CD are epithelioid granulomas, bowel wall thickening, discontinuous inflammation, mucin preservation at the edge of an ulcer, relatively unchanged crypt architecture or patchy crypt atrophy and changes in the intensity of distribution of lamina propria inflammatory cells [11, 17].

When treating IBD patients the focus is always to induce and maintain clinical remission with progressive intensification of therapy as the disease worsens [18]. Current treatment options include a combination of, antibiotics, vitamin support, immunomodulators, corticosteroids, 5-aminosalicylates, biologic therapies and surgery [11, 19]. It is not uncommon for CD patients to undergo multiple surgeries to control reoccurring disease while in UC, removal of the colon and rectum will not only eliminate disease but also removes the risk of colorectal cancer developing.

1.3 GENETIC RISK FACTORS

IBD is a complex polygenic disorder with complex inheritance. An individual's genetic background is known to not only influence disease onset, but also the phenotype, the location and the disease course [20]. Using genome-wide association studies (GWAS) and subsequent meta-analyses a total of 240 loci have been identified as reaching significance thresholds and being associated with disease development [21]. While many of these loci contain multiple genes, others contain no genes. Of the 240, 110 are known to contribute to both phenotypes, 30 are Crohn's disease specific loci and 23 are ulcerative colitis specific [22]. Notably many loci overlap with the susceptibility to other inflammatory diseases such as ankylosing spondylitis and psoriasis, while others have been implicated in mechanisms such as, bacterial sensing, epithelial defence, cytokine signalling and autophagy [22]. Of the polygenic disorders IBD is unusual in that it has provided replicate genetic susceptibility using both GWAS and linkage studies [10].

Combining GWAS studies and the greater concordance in monozygotic twins for CD than UC it appears the contribution of genetic factors may be more important in CD than UC [20, 23]. The general theme emerging for CD involves one of defective processing of intracellular bacteria with innate immunity and autophagy genes such as NOD2, IRGM, ATG16L1 being CD specific associations. While in UC, defects in epithelial barrier function highlighted by gene associations with HNF4A, LAMB1, CDH1 and GNA12 are thought to influence disease development [24].

The many number of genes that confer susceptibility to both CD and UC such as those involved in IL23/Th17 signalling (IL23R, IL12B, JAK2, TYK2, STAT3, IL10,

IL1R2, REL, CARD9, NKX2.3, ICOSLG, PRDM1, SMAD3 and ORMDL3) suggest common pathways may also be responsible for disease pathogenesis [24]. Considered important to the development of these diseases are the missense polymorphisms, such as single nucleotide polymorphisms(SNPs) that mediate mRNA changes that result in protein structure or function alterations. Noteworthy, is many of these protein-coding regions are often distant from their gene regulatory regions [25].

1.4 ENVIRONMENTAL RISK FACTORS

The recent emergence of IBD in developing countries combined with the variability of disease presentation with geographical location suggests that environmental influences may contribute to disease development. Urbanisation of societies and adoption of western culture has led to changes in diet, increased antibiotic use, improved hygiene, better vaccination rates and increased pollutant exposure, all of which have been highlighted as potential risk factors, however most are poorly defined [26, 27].

Smoking and appendectomy are two well established risk factors for IBD, which impact on the course of the disease but fail to correlate with the incidence or prevalence of the disease. For instance, Canada and Sweden have a high incidence of CD with a low prevalence of smokers while Africa and Asia have a high prevalence of smokers with a low incidence of CD [27]. For CD patients, smoking results in a worse disease course and more recurrent disease flare-ups while in UC, smoking is protective for development of the disease and UC patients who smoke experience a milder disease course [28-32].

For UC patients, appendectomy before the age of 20 is considered protective. Patients diagnosed after appendectomy have a more limited disease extent with fewer clinical relapses when compared to patients with an intact appendix [33-36]. The protective nature of appendectomy on CD remains unclear with several studies showing a positive effect [34] while others demonstrate no association [37-39]. Neither smoking or appendectomy have been shown to be necessary or causative for the development of IBD [40].

1.5 THE INNATE IMMUNE SYSTEM

1.5.1 INTESTINAL EPITHELIAL BARRIER

The epithelial cell layer separates the luminal contents of the bowel from the immune cells of the lamina propria and is often the first site of exposure for many of the environmental and pathogenic factors that contribute to disease pathology. The development of the epithelial cell layer begins with multipotent stem cells located within the epithelial invaginations known as the crypts of Lieberkühn [20]. Intermingling with multipotent stem cells are paneth cells, which have originated from undifferentiated stem cells. Under the direction of Wnt and Notch signalling pathways epithelial stem cells differentiate into absorptive enterocytes, mucus-producing goblet cells and hormone-secreting enteroendocrine cells and migrate from the crypts to the intestinal villi [41].

The integrity of the epithelial barrier is maintained by intercellular junctions on the lateral cell surface which anchor cell-cell contacts to the actomyosin cytoskeleton [42]. Movement through the junctions is by paracellular permeability based on size, osmotic or electrical gradients and is controlled by a variety of different host, dietary and microbial factors. Cytokines like IL-10 and transforming growth factor-β (TGF-β) enhance tight junction while inflammatory cytokine like IL-1β, IL-4, IL-13, tumor necrosis factor-α (TNF-α) and IFN-ɣ degrade tight junction permeability by altering the claudin composition [20, 43, 44]. Increased epithelial permeability is a well-established consequence of mucosal inflammation and considered an important contributor to the development of gastrointestinal diseases. Remarkably, prior to disease relapse CD patients demonstrate increased intestinal permeability, [45, 46] while UC

patients experience more disease relapses with increased intestinal permeability [47].

1.5.2 PATHOGEN RECEPTORS OF THE INNATE IMMUNE SYSTEM

The innate immune system is the "danger sentinel" of the gut and maintains mucosal homeostasis by using an array of germline-encoded pattern recognition receptors to continually scan the cellular and luminal environments [48]. Pattern recognition receptors primarily recognise conserved microbial molecules known as pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs) released in response to stress, tissue damage and necrotic cell death [49-51]. Cells involved in first-line defence mechanisms such as dentritic cells, epithelial cells, monocytes, macrophages and neutrophils are all known to express pattern recognition receptors [48].

Membrane bound pattern recognition receptors, such as Toll-like receptors (TLRs) and C-type lectin receptors (CLRs) are responsible for monitoring the extracellular milieu and endosomal compartments for PAMPs and DAMPs. While cytoplasmic surveillance is executed by NOD-like receptors (NLRs), pyrin and HIN domain containing (PYHIN) family members, RIG-1-like receptors (RLRs) and several cytosolic nucleic acid sensors [52]. Membrane and intracellular receptors work in concert with one another to orchestrate an effective immune response against a potential pathogenic insult. Often an intact microbial pathogen will contain multiple PAMPs which trigger the sequential detection by a number of receptors in different subcellular compartments [53].

Pattern recognition receptors have the ability to promote the secretion of proinflammatory cytokines, transcription mediators, and initiate pathways

responsible for pathogen neutralisation and elimination [54]. In addition, some receptors are known to form the structural backbone of the multimolecular complex known as the inflammasome.

1.6 THE INFLAMMASOME COMPLEX

The inflammasome complex is a core component of the inflammatory response and its activation enhances the maturation of proIL-1β and proIL-18 to their biologically active IL-1β and IL-18 forms [48]. Transcription of proIL-1β is induced by TLR and CLR stimulation via the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) transcription pathway, whereas proIL-18 is constitutively expressed and its expression is increased after receptor activation [55, 56]. For activated macrophages and monocytes of the lamina propria inflammasome maturation of IL-1β and IL-18 is crucial for cytokine secretion [57].

In the intestine, the inflammasome can also promote an inflammatory form of cell death, known as pyroptosis. Pyroptosis halts the replication of intracellular pathogens by destroying the infected immune cell and exposing the surviving bacteria to circulating phagocytes and neutrophils [58]. Both canonical (caspase-1) and non-canonical (caspase-11) inflammasome pathways are able to induce pyroptosis, however caspase-11 does not produce mature IL-1β or IL-18. Caspase-11 induced pyroptosis is thought to occur upstream of canonical inflammasomes in response to lipopolysaccharides (LPS) sensed in Gram-negative bacteria. Both mechanisms are considered important for microbial defences in the gut [58, 59].

In addition to inflammasome-dependent production of IL-1β, several cell specific inflammasome-independent processes exist for the activation of IL-1β. During acute inflammation, neutrophil derived serine proteases, proteinase 3, elastase and cathepsin G can process proIL-1β to biologically active IL-1β [60-63].

1.6.1 FORMATION OF A NOD-LIKE RECEPTOR PROTEIN (NLRP) INFLAMMASOME COMPLEX

In general, the NOD-like receptor protein (NLRP) inflammasome complex consists of a nucleotide-binding oligomerisation domain (NOD)-like receptor (NLR) protein, a caspase and often an adaptor protein known as apoptosis-associated speck-like protein containing a CARD (ASC) [48, 64]. Several receptors from the NLR family, NLRP1, NLRP2, NLRP3, NLRC4, NLRP6, NLRP7 and NLRP12 (Figure 1-2) have all shown the ability to form the structural backbone of an inflammasome complex. The ASC adaptor protein is identical for all inflammasomes and contains two transduction domains, a pyrin domain (PYD) domain and a caspase recruitment domain (CARD) domain [65].

Formation of a NLRP inflammasome is initiated by ligand activation of the receptor protein and this causes the NLR proteins to oligomerise through their nucleotide binding and oligomerisation (NACHT) domains (Figure 1-3). This oligomerisation creates a platform of NLR^{PYD} molecules at the N-terminal and through NLR^{PYD}/ASC^{PYD} interactions nucleates helical ASC clusters to form an ASC filament structure. The aggregation of multiple ASC^{CARD} molecules promotes ASC^{CARD}/caspase-1^{CARD} interactions, which in turn brings caspase domains into close proximity for dimerisation, *trans*-autocleavage and activation [65]. The binding of ASC to both the NLR protein and caspase-1 is facilitated by a 23-residual linker which orientates ASC^{PYD} and ASC^{CARD} back to back hence preventing steric interference of binding sites, while enhancing binding partner prospects [66]. ASC is sequestered in the nucleus but rapidly translocates to the cytoplasm upon stimulation where it participates in inflammasome formation

[67]. Interestingly, inflammasome formation can be abolished by preventing the cellular redistribution of ASC [67].

Caspase-1 is synthesised as an inactive, monomeric zymogen (procaspase-1) and initially is cleaved into a p35 fragment containing a CARD and p10 fragment. Autoproteolysis results in the generation of a large p20 subunit and a small p10 subunit and the removal of the N-terminal CARD domain. Dimerisation of caspase molecules (p20 and p10) results in the catalytically active caspase-1 enzyme (Figure 1-4) [68, 69]. Inflammasome activated caspase-1 cleaves its substrates, proIL-1β and proIL-18 at recognition sites adjacent to aspartic acid residues, resulting in mature IL-1β and IL-18 [64].

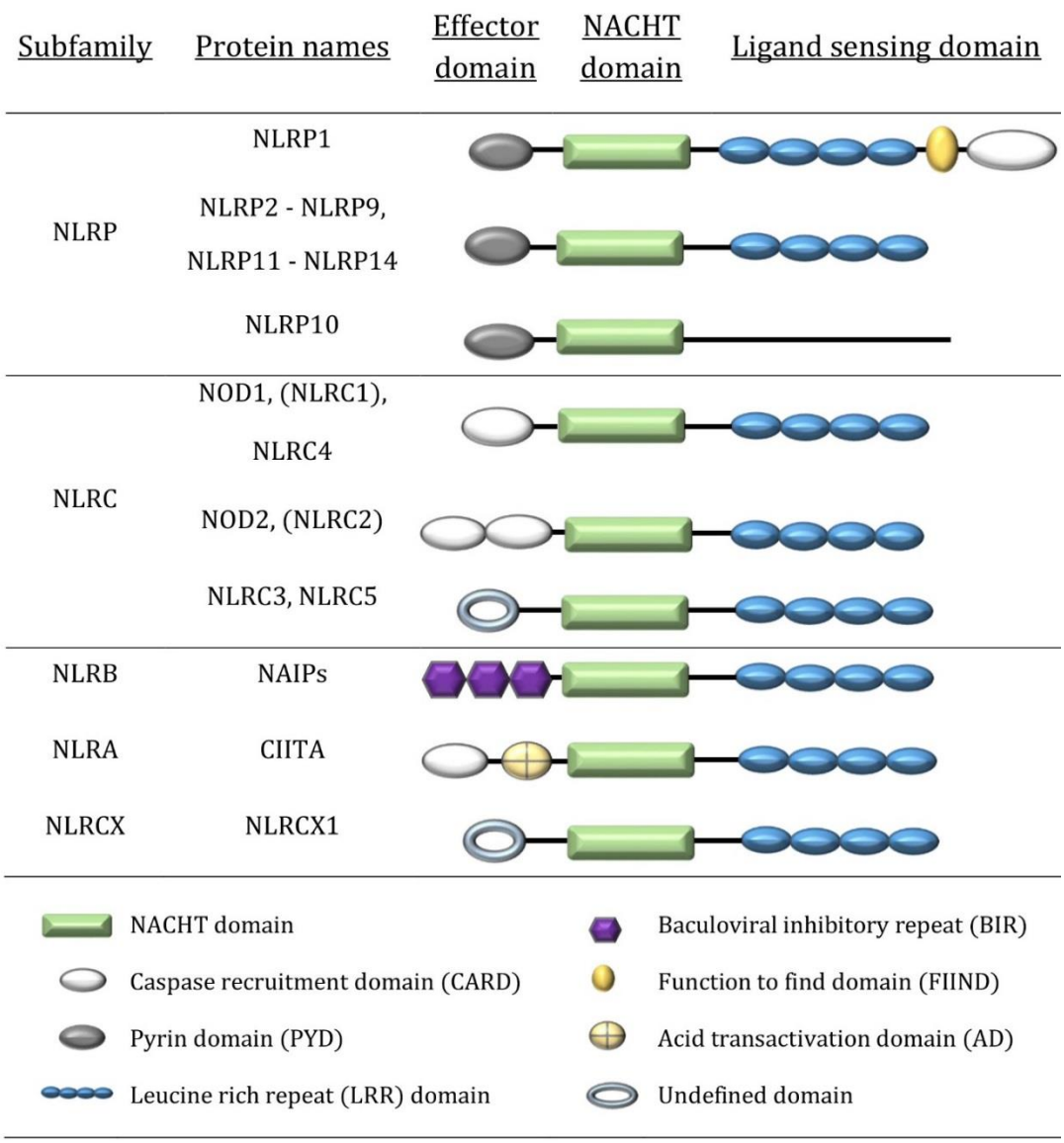


Figure 1-2: Structure of the human NOD-like receptor subgroups

The NOD-like receptor (NLR) family comprises 23 human members [20, 70]. All NLR proteins contain a central nucleotide binding and oligomerisation (NACHT) domain flanked by a C-terminal LRR domain and N-terminal effector domain. The NACHT domain facilities self-oligomerisation and has ATPase activity. The N-terminal domain participates in protein-protein interactions while the LRR domain is involved in ligand recognition. Subgroup classification is based on the structure of the N-terminal effector region, which generally comprises a CARD, PYD or BIR domain. The NLRP1, NLRP2, NLRP3, NLRC4, NLRP6, NLRP7 and NLRP12 receptors have all shown the ability to form inflammasome complexes.

In contrast to other members of the NLRP subfamily, NLRP1 contains both a function-to-find (FIIND) and CARD domain at the C-terminal, and a PYD domain at the N-terminal [71] (Figure 1-2). Given that NLRP1 contains two signal transduction domains (PYD and CARD) it can activate caspase-1 through its C-terminal CARD domain without the need for the ASC adaptor protein, however ASC has been shown to greatly enhance inflammasome formation and IL-1β processing [72]. The FIIND domain is a highly conserved protein region and based on amino acid sequencing is only present in two human proteins, NLRP1 and the caspase recruitment domain family, member 8 (CARD8) protein [73]. CARD8 is thought to function as an adaptor molecule that negatively regulates NF-κB activation, caspase-1 dependent IL-1β secretion and apoptosis, and is often overexpressed in many types of cancers [74-76].

NLRP1 inflammasome formation is strictly dependent on autolytic proteolysis within the FIIND domain and after cleavage, the two fragments remain associated to form a processed NLRP1. Dimers of ASC joined by ASC^{PYD}/ASC^{PYD} are recruited to the C-terminal NLR^{CARD} domain and bind via NLR^{CARD}/ASC^{CARD} interactions. This is in contrast to other NLRP proteins which recruit ASC to the N-terminal PYD domain and bind via NLR^{PYD}/ASC^{PYD} interactions to form the inflammasome complex. Subsequently, caspase-1 through its CARD domain interacts with ASC^{CARD} which leads to dimerisation, *trans*-autocleavage and activation of caspase-1 and IL-1β, IL-18 processing [77]. The formation of ASC filaments in the activation of the NLRP1 inflammasome remain to be defined.

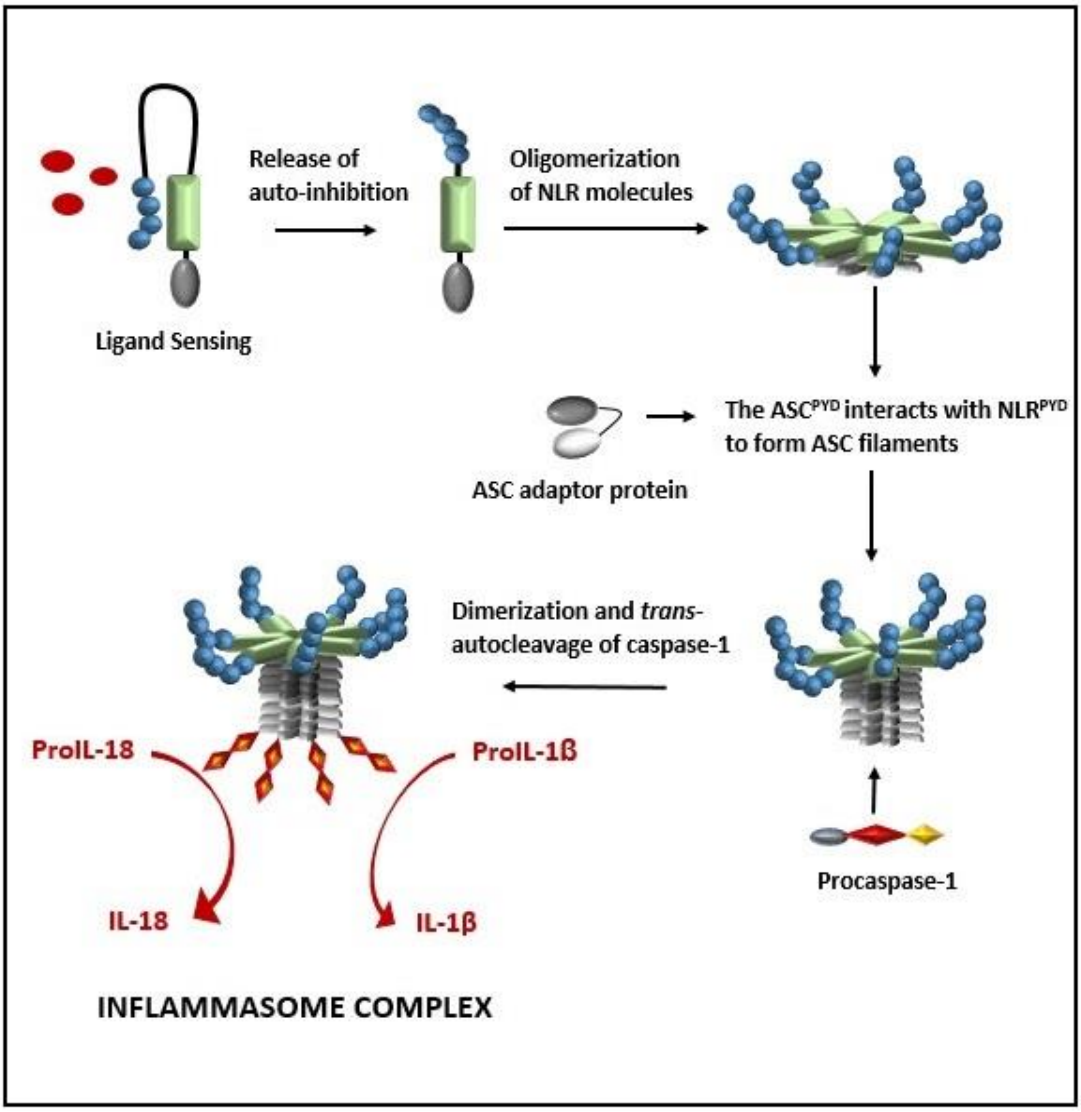


Figure 1-3: Formation of a NOD-like receptor protein inflammasome containing an N-terminal pyrin domain

Formation of a nucleotide-binding oligomerisation domain (NOD)-like receptor protein (NLR) inflammasome is initiated by ligand activation of the NLR protein. This causes the NLR proteins to oligomerise through their NACHT domains to create a platform of NLR^{PYD} molecules at the N-terminal and through NLR^{PYD}/ASC^{PYD} interactions, nucleates helical ASC clusters to form a filament ASC structure. The aggregation of multiple ASC^{CARD} molecules promotes ASC^{CARD}/caspase-1^{CARD} interactions which in turn brings caspase domains into close proximity for dimerisation, *trans*-autocleavage, activation and the processing of proIL-1β and proIL-18 to their biologically active forms, IL-1β and IL-18 respectively.

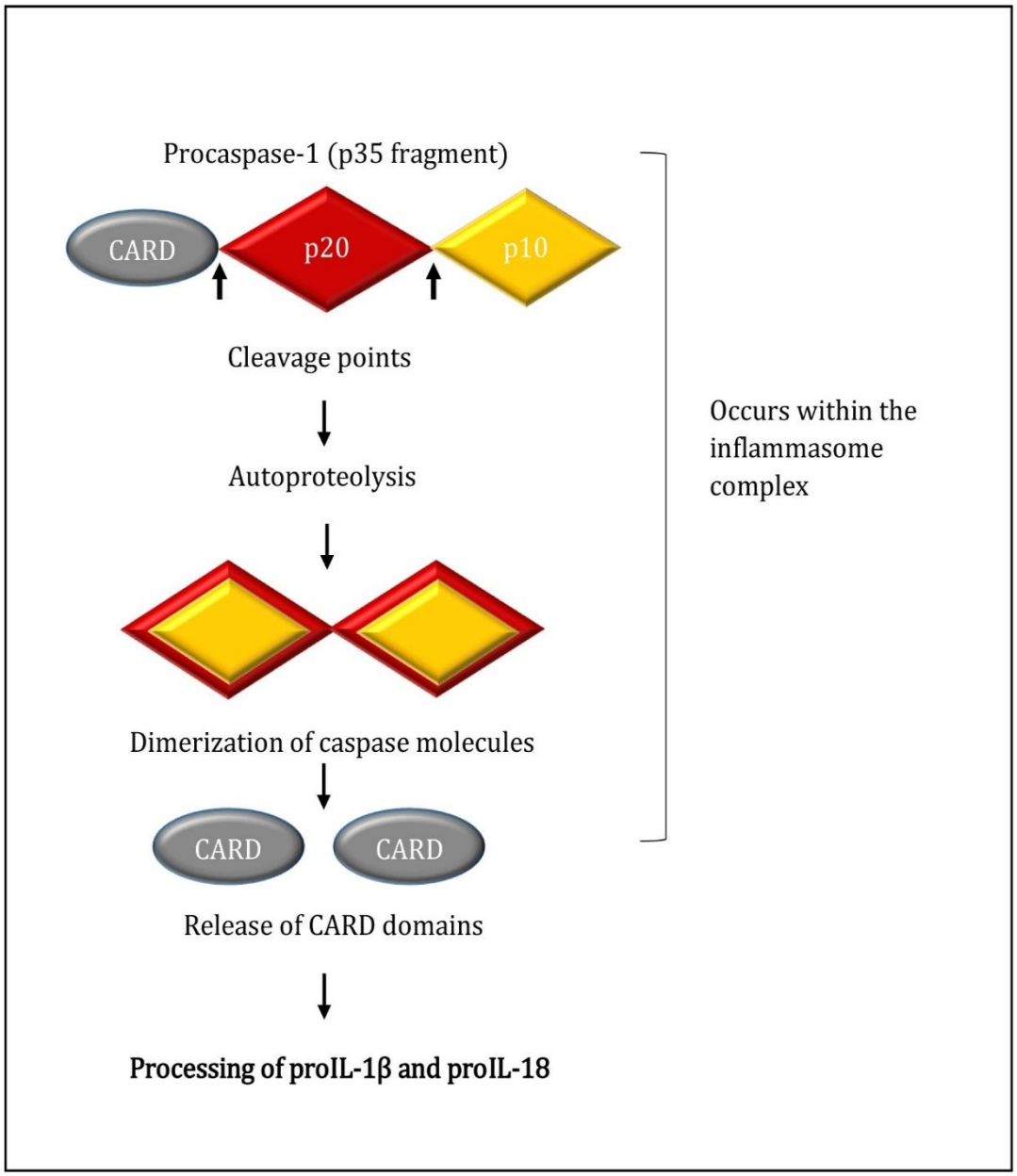


Figure 1-4: The mechanism for the inflammasome mediated catalytic conversion of procaspase-1 to caspase-1

Caspase-1 is initially synthesised as the inactive monomeric zymogen, procaspase-1. Binding of the procaspase-1^{CARD} to ASC^{CARD} filaments on the inflammasome complex results in the cleavage of procaspase-1 into a p35 fragment containing a CARD and a p10 fragment. Dimerisation of the p10 and p20 and the removal of the procaspase-1^{CARD} domain produces catalytically active caspase-1.

1.6.2 STRUCTURE AND FORMATION OF A PYRIN AND HIN DOMAIN (PYHIN) INFLAMMASOME COMPLEX

Two receptor in the pyrin and hematopoitic interferon-inducible nuclear proteins (HIN) (PYHIN) receptor family, absent in melanoma 2 (AIM2) and interferon inducible protein 16 (IFI16) have shown the ability to form inflammasome complexes [68, 78] (Figure 1-5). Similar to NLRP inflammasomes, PYHIN inflammasomes, such as AIM2, upon ligand activation oligomerise through their PYD domains to form a platform of $AIM2^{PYD}$ molecules, which preferentially associates with ASC^{PYD} to form ASC filaments. The flexibly linked ASC^{CARD} clusters along the ASC^{PYD} to form a platform for the binding of caspase-1^{CARD}. Similar to other NLRP inflammasomes, the ASC filament structure forms the main body of the inflammasome. The interaction of ASC^{CARD}/caspase-1^{CARD} brings caspase domains into close proximity for dimerisation, *trans*-autocleavage and activation, and the subsequent maturation of IL-1β and IL-18 [65].

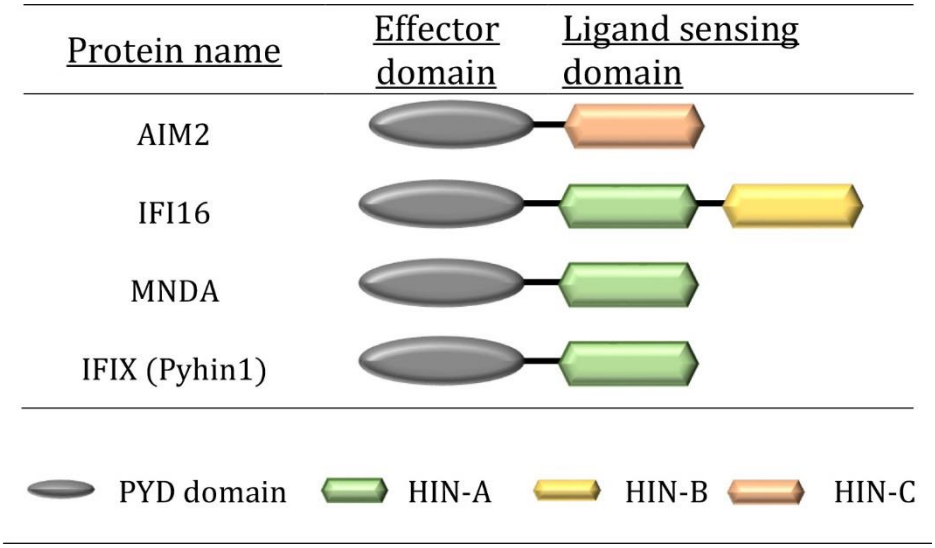


Figure 1-5: Structure of the human pyrin and HIN domain (PYHIN) family

The human pyrin and hematopoitic interferon-inducible nuclear proteins (HIN) (PYHIN) family of receptors comprises 4 members including, the interferon inducible protein 16 (IFI16), absent in melanoma 2 (AIM2), myeloid nuclear differentiation antigen (MNDA) and interferon inducible protein X [IFIX (Pyhin1)], while mouse contains 11 confirmed members [79]. All members consist of an N-terminal pyrin domain (PYD) domain attached to one or more hemopoietic interferon-inducible nuclear protein (HIN-200) domains at the C-terminal. Three distinct forms of HIN-200 have been characterised (HIN-A, -B and -C) and are classified according to specific consensus motifs [80].

1.7 LIGAND SENSING OF INFLAMMASOME COMPLEXES

Depending on the type of receptor protein in the complex, inflammasomes have the ability to respond to a wide array of pathogens and cellular danger signals. The LRR domains of the NLRP receptors and the HIN200 domains of the PYHIN receptors are thought to be involved in ligand interactions, however direct binding of an activating ligand to a receptor has only been demonstrated for the AIM2 and IFI16 inflammasomes.

1.7.1 THE NLRP1 INFLAMMASOME

The NLRP1 inflammasome was one of the first inflammasomes to be described however, efforts to unravel the processes that lead to activation have been hampered by species variations in the *NLRP1* gene. In humans the *NLRP1* gene is singular, while in mouse the gene encoding *Nlrp1* is polymorphic with three homologs, *Nlrp1a, Nlpr1b* and *Nlrp1c* [68]. Furthermore, the structure of mouse *Nlrp1* lacks the N-terminal PYD domain found in human NLRP1 and five different strain specific *Nlrp1b* alleles exist in inbred mice [81].

Nlrp1 is activated mainly by lethal toxin (LeTx) produced by *Bacillus anthracis* with variations in *Nlrp1b* providing sensitivity or resistance to the toxin [82]. LeTx is a bipartite toxin consisting of a protective antigen binding subunit and a catalytic lethal factor moiety. Binding of the protective antigen to anthrax binding sites translocates lethal factor into the host cytosol where it cleaves the N-termini of mitogen-activated protein kinase (MAPK) thereby disrupting cell signalling pathways. Initially lethal factor blocks cytokine production from numerous cell types, inhibits chemotaxis of neutrophils, induces apoptosis in activated macrophages and later induces cytokine-independent shock and death [83].

Caspase-1 and IL-1β deficient mice are more susceptible to *B.anthracis* infection indicating IL-1β production via the NLRP1b inflammasome is more important than ASC independent pyroptosis in the host protective response to *B.anthracis* [69, 83].

More recently, NOD2 has been linked to NLRP1 dependent sensing of bacterial muramyl dipeptide (MDP) and *B.anthracis* in activated cells where it produces a NOD2-NLRP1 inflammasome complex [84]. NOD2 is a known intracellular sensor of MDP and has the ability to contribute to the induction of NF-κB and MAPK transcription factors, however TLRs are much more effective in triggering these responses [85]. The absence of NOD2 prevents *B. anthracis* induced IL-1β secretion but has little effect on the transcription of proIL-1β indicating the importance of the NOD2-NLRP1 association in host defences against B. *anthracis* [84].

1.7.2 THE NLRP3 INFLAMMASOME

The NLRP3 inflammasome has the ability to activate upon exposure to a wide range of whole pathogens, environmental irritants and structurally diverse DAMPs and PAMPs [48, 49, 86].

While the mechanisms are not yet fully understood it is thought that activation of NLRP3 occurs in response to host derived factors that are altered by these agents. While several models have been proposed for the activation of NLRP3 none have been found to be unified for all activating agents. The proposed mechanisms include;

1. K^+ efflux [87]

2. The generation of mitochondrial derived reactive oxygen species (mROS) [88]
3. Phagolysosomal destabilisation and the release of cathepsins [89]
4. The release of mitochondrial DNA or the mitochondrial phospholipid cardiolipin [90-92]
5. Translocation to the mitochondria [88, 93, 94]

To add to the controversy, membrane permeation, phagolysosmal destabilisation, mitochondrial damage and ROS production are all interrelated cellular events making the distinction between bystander and causative activation events complicated.

In resting cells, the basal expression of *NLRP3* is insufficient for inflammasome activation and consequently two signals are required for the activation of the NLRP3 inflammasome [95, 96]. The first signal is the NF-κB mediated transcription of *NLRP3* and *proIL-1β* from stimulation of TLR antagonists or cytokines such as TNF-α and IL-1β. The second signal is the ligand activation step which culminates in the activation of caspase-1 and the maturation of IL-1β and IL-18 [49, 97]. The enhanced effect of guanylate binding protein (GBP5) on Nlrp3 inflammasome assembly in response to bacteria and soluble but not crystalline inflammasome priming agents raises the possibility of agent specific cofactors being required for inflammasome activation [98].

Particulate matter such as aluminium, silica, monosodium urate (MSU), calcium pyrophosphate dehydrate crystals, cholesterol and amyloid β enters the cell by means of phagocytosis [89, 99-101]. The destabilisation of the phagolysosmal membrane and the release of the cysteine protease cathepsins B into the cytosol is thought to also trigger NLRP3. Inhibitors of cathepsins B have been shown to

prevent caspase-1 activation induced by *N. gonorrhoeae [102].* Interestingly, cathepsins deficient mice show minimal defects in the activation of NLRP3 in response to particulate matter, suggesting other off target effects may exist [103]. More recently, mitochondrial dysfunction and activation of the NLRP3 inflammasome has been an area of intense research and much speculation. mROS are produced in response to cell stresses such as, hypoxia, starvation, pathogen infection, and growth factor stimulation or membrane damage [104]. The release of mROS and oxidised mitochondrial DNA have both been shown to activate the NLRP3 inflammasome [90, 105]. Interruption of ROS production using inhibitors blocks NLRP3 activation suggesting ROS production upstream is necessary for NLRP3 activation [105-107].

It has been proposed that NLRP3 associates with the mitochondria upon activation [93, 105] and when exposed to non-crystalline activators, recruitment from the cytosol to the mitochondria is mediated by the mitochondrial anti-viral signalling protein (MAVS) [94]. MAVS is also known as a mitochondrial adaptor protein and plays a crucial role in RLR receptor signalling pathways leading to type 1 IFN induction and NF-κB activation [108]. MAVS is thought to directly associate with the N-terminus of NLRP3 to promote optimal inflammasome formation [94]. Consistent with a role for MAVS in NLRP3 activation, MAVS deficient mice exposed to dextran sodium sulphate (DSS) induced colitis fail to upregulate IL-1β [109].

Other work on mitochondrial dysfunction has demonstrated a ROS independent activation of *Nlrp3* induced by the antibiotic linezolid whereby the mitochondrial specific lipid cardiolipin binds to *Nlrp3* leading to the maturation of IL-1β [92]. Cardiolipin is a phospholipid exclusively found in the inner mitochondrial

membrane of eukaryotic cells. Cardiolipin plays a critical role in the activation of caspase-8 and caspase-3 in the apoptotic cell death pathway, which raises the possibility that the inflammasome pathways are linked to the apoptosis pathways by processes that control mitochondrial homeostasis.

In addition, agents that induce NLRP3 activation, such as nigericin have demonstrated an ability to disrupt mitochondrial homeostasis by reducing the intracellular concentration of the coenzyme NAD^+. Low NAD^+ inactivates the α-tubulin deacetylase sirtuin 2 (SIRT2) and causes the accumulation of acetylated α-tubulin. Excess acetylated α-tubulin mediates the microtubule transport of mitochondria, which drives the apposition of ASC on the mitochondria to NLRP3 on the endoplasmic reticulum. Microtubule transport of organelles creates optimal sites for signal transduction between ASC and NLRP3 and directs activation of NLRP3. Work using inhibitors of tubulin polymerisation have demonstrated suppression of IL-1β [93].

Early work investigating caspase-1 activation by the NLRP3 inflammasome showed that K^+ efflux accompanies NLRP3 activation [106, 110] and a high extracellular concentration of K^+ blocks the activation of not only the NLRP3 inflammasome but also the NLRP1, NLRC4 and AIM2 inflammasomes [111, 112]. ATP levels have been linked to K^+ efflux, such that high extracellular ATP levels engage the ATP-gated purinergic $P2X_7$ receptor promoting the formation of the pannexin-1 pore, which induces K^+ efflux [97, 113]. Previous work by Muñoz-Planillo [87] has shown that ROS generation, opening of the pannexin-1 pore and K^+ efflux all occur upon stimulation with a variety of bacterial pore-forming toxins, nigericin, ATP and particulate matter. However in contrast to others the permeation of the cell membrane to K^+ and Na^+ was found to be the only

common step induced by all NLRP3 antagonists and the primary activity that was necessary and sufficient for caspase-1 activation. In addition, cytosolic K^+ efflux was found to be specific to NLRP3 activation and was shown not to play a role in the activation of AIM2. These results await further clarification by other independent researchers.

1.7.3 THE NLRC4 INFLAMMASOME

The NLRC4 inflammasome has been well characterised in the mouse system and plays an important role in the detection of pathogenic bacteria [68]. The pathogenicity of a bacteria is reliant on functional secretion systems including the type III and IV which act as needle-like structures delivering virulent factors into the host's cytosol. NLRC4 is activated by two critical components of pathogenic bacteria, a sequence motif found in the basal rod components of the type III (T3SS) and IV (T4SS) bacterial secretion systems, and a similar sequence motif found in flagellin, which is a component of their flagellum apparatus [114, 115]. NLRC4 has been shown to detect basal rod components in *Salmonella typhimurium*, *Legionella pneumophila*, *Burkholderia pseudomallei, Escherichia coli, Shingella flexneri, Pseudomonas aeruginosa [116, 117]* and leaked cytosolic flagellin from *Listeria monocytogenes, Salmonella typhimurium, Pseudomonas aeruginosa* and *Legionella pneumophila [115, 118, 119].*

Activation of the NLRC4 inflammasome involves the initial binding of a receptor protein from the neuronal apoptosis inhibitory protein (NAIP) subfamily of NLRs to the activating ligand. NAIP receptor proteins differ from other NLRs in that they contain multiple BIR domains at the N-terminus instead of a CARD or PYD domain (Figure 1-2). In humans, only one *NAIP* homolog is expressed, whereas

in mice, the *NAIP* locus is polymorphic and seven paralogs of *Naip* (*Naip1-Naip7*) exist [117]. Human NAIP and its mouse ortholog, Naip1 recognise cytosolic T3SS needle proteins, Naip2 binds T3SS rod components while Naip5 and Naip6 bind directly to bacterial flagellin [117, 119]. Binding of a NAIP protein to a bacterial motif leads to the formation of the NAIP-NLRC4 inflammasome complex and activation of caspase-1.

Interestingly, in human U937 monocyte derived macrophages NLRC4 activation does not occur in response to flagellin or T3SS rod protein but occurs in response to the T3SS subunit Cprl from *Chromobacterium violaceum,* which raises the possibility that other accessory proteins may be involved in activation of the human NLRC4 inflammasome [119].

1.7.4 THE AIM2 AND IFI16 INFLAMMASOME

Both the cytosolic AIM2 receptor and the nuclear IFI16 receptor directly bind their activating ligand double stranded DNA (dsDNA) via the C terminal HIN200 domain [120-122]. Non-sequence specific binding occurs at multiple sites along the dsDNA and is through electrostatic attractions between the positively charged HIN domain residues and the dsDNA sugar phosphate backbone [123]. The mechanisms that enable AIM2 and IFI16 to respond to viral, bacterial, mammalian and synthetic dsDNA while remaining unresponsive to self DNA are still unclear [68, 124].

Work using *Aim2* deficient mice have demonstrated an essential role for AIM2 in the recognition of viruses and bacteria by the detection of cytosolic dsDNA. When compared to WT mice, *Aim2-/-* mice experience higher mortality rates, higher bacterial loads and decrease production of caspase-1 generated cytokines after infection with *Fransicella tularenis,* suggesting AIM2 is necessary for detection of *Fransicella tularenis* [125, 126]. Similarly, mouse macrophages deficient for *Aim2* show an impaired ability to recognise not only *Fransicella tularenis,* but also vaccinia virus, murine cytomegalovirus (mCMV) with only partial recognition of *Listeria monocytogenes* being demonstrated [120, 127].

1.7.5 THE NLRP6, NLRP7 AND NLRP12 INFLAMMASOMES

In addition to the well-known NLRP1, NLRP3, NLRC4, AIM2 and IFI16 inflammasomes NLRP6, NLRP7 and NLRP12 have shown an ASC-dependent activation of caspase-1. However, the signals that activate the NLRP6 and NLRP12 inflammasomes are yet to be determined [128].

Indeed, two independent studies have reported caspase-1 activation and IL-1β release in Nlrp6 deficient mouse macrophages in response to ATP and LPS, which suggest the triggers that activate the NLRP6 inflammasome are different to those that activate the NLRP3 inflammasome [129, 130]. Recently NLRP7, which is not expressed in mice, was shown to form an ASC-dependent inflammasome in human macrophages in response to microbial acylated lipopeptides [131].

1.8 REGULATION OF THE INFLAMMASOME COMPLEX

The potent inflammatory cytokines, IL-1β and IL-18 and the pyroptosis pathway all have the potential to cause tissue damage and disrupt an effective adaptive immune response. The mechanisms that lead to maturation of IL-1β and IL-18 are tightly controlled at several levels and multiple checkpoints along this process ensuring response appropriate levels.

In most cells the basal levels of many of the inflammasome constituents is insufficient for inflammasome formation. Consequently, the expression of the inflammasome components is regulated by NF-κB induced transcription and requires sensitisation by a TLR or CLR ligand or stimulus from cytokine signalling pathways [49, 50]. In contrast to most cytokines, IL-1β and IL-18 are produced as inactive zymogens requiring caspase-1 cleavage between Asp and Ala for maturation [55, 132]. The synthesis of precursor cytokines requiring activation prevents aberrant secretion of the leaderless IL-1β and IL-18 cytokines. Serine proteinases such as cathepsins G, elastase and in particular proteinase 3 found in neutrophils have also been shown to cleave proIL-1β to active IL-1β. While in monocytes autocrine production of ATP can activate caspase-1 and cleave proIL-1β, thereby releasing IL-1β by transcription only [60]. Worth noting is that during acute inflammatory conditions non-canonical maturation of IL-1β can also occur via caspase-11 and the NLRP3 inflammasome [58].

1.8.1 REGULATION BY AUTOINHIBITION OF THE LIGAND SENSING DOMAIN

For most of the receptor proteins autoinhibition of the ligand sensing domain prevents unproductive intramolecular interactions by providing a tight on-site repression of the protein in the absence of a suitable activating ligand. For NLRP1, NLRP3, NLRP12 and NLRC4 receptors autoinhibition is achieved by the association of two chaperone proteins, ubiquitin ligase-associated protein (SGT1) and heat-shock protein 90 (HSP90) to the LRR domain. Upon ligand sensing, SGT1 and HSP90 dissociate resulting in a conformation change within the protein which favours the recruitment of the ASC adaptor protein [133]. Whether autoinhibition of the sensing region occurs for the NLRP6 protein remains to be determined. For the PHYIN subfamily autoinhibition is provided by the molecular interactions between the PYD and HIN-200 domain and binding of DNA releases this autoinhibition [123].

1.8.2 PRIMING EVENTS THAT REGULATE ACTIVATION

Specific priming events are known to regulate the activation of inflammasomes. The K-63 specific deubiquitinating enzyme BRCC3 mediates the deubiquitylation of NLRP3, which has recently been shown to occur in response to pattern recognition receptor stimulation [134]. Similarly, and as mentioned above, GBP5 enhances NLRP3 assembly in response to bacterial but not crystalline agents [98]. A priming event involving the phosphorylation of Ser533 by kinases like PKCδ is necessary before *Salmonella typhimurium* can activate the NLRC4 inflammasome. The phosphorylation of Ser533 is thought to result in a conformation change within the NLRC4 protein [135].

The anti-apoptotic proteins B-cell lymphoma 2 (Bcl-2) and B-cell lymphoma extra-large (Bcl-X_L) have been shown to regulate the NLRP1 inflammasome. By associating with NLRP1 via their loop domains Bcl-2 and Bcl-X_L are able to suppress caspase-1 activation and IL-1β processing [136, 137]. Similar experiments for NLRP3 in Bcl-2 deficient macrophages have shown more caspase-1 processing while Bcl-2 overexpressing macrophages demonstrated less caspase-1 processing suggesting NLRP3 may also be regulated by Bcl-2 protein [90].

Activation of the NLRP3 inflammasomes is thought to be influenced by K^+ levels, indeed low intracellular K^+ level enhance caspase-1 activation [106, 110]. Similarly, high extracellular K^+ levels block IL-1β release from the NLRC4 and AIM2 inflammasome suggesting the regulatory effect of K^+ may be extended to other inflammasome complexes [125, 138]. Interestingly, the levels of extracellular K^+ need to block IL-1β release for the NLRP3 inflammasome is less than that needed for the NLRC4 or AIM2 complex, while for the NLRP7 inflammasome high K^+ levels only slightly reduced IL-1β release [131]. More work is needed to exclude off target effects and to determine the reasons for inflammasome specific thresholds to K^+ levels.

1.8.3 REGULATION BY POPS AND COPS

In humans, pyrin only proteins (POPs) and CARD-only proteins (COPs) regulate the inflammasome at the level of death fold interactions. With the exception of caspase-12, POPs and COPs are lacking from the mouse genome which suggests humans have evolved more complex inflammasome regulatory systems [139]. The POPs include, POP1 (also known as PYDC1) and POP2 (also known as

PYDC2) and both inhibit PYD interactions between the receptor protein and the ASC adaptor molecule. POP1 shows a higher homology to ASC^{PYD} than POP2 and therefore inhibits inflammasome formation by sequestering ASC from other inflammasome forming NLRs [140]. POP2 is surprisingly similar to the PYD domain of NLRP2 and NLRP7 and is thought to interact with other NLR^{PYD} proteins thereby preventing inflammasome formation [141]. Both POP1 and POP2 can prevent NF-κB activation [140, 141].

The COPS proteins consists of several members including, CARD16 (also known as pseudo-ICE or COP1), CARD17 (also known as INCA), CARD18 (also known as ICEBERG), caspase-12s and Nod2-S.[142] COP proteins act as decoy inhibitors and sequester procaspase-1 via CARD-CARD interactions thereby preventing its activation in the inflammasome. For example, CARD 17 is upregulated by IFN-ɣ in the monocytic cell lines THP-1 and U937 and interacts with procaspase-1 to supress IL-1β processing and release in LPS stimulated macrophages [143].

1.8.4 REGULATION BY TYPE I INTERFERONS

Type I interferons restrict IL-1β production by two distinct mechanisms. Depending on the cell type, type I interferons through the STAT3 signalling pathway can induce autocrine and paracrine production of the anti-inflammatory cytokine IL-10 which inhibits the synthesis of proIL-1β and proIL-18. Additionally, type I interferons signalling through the STAT1 transcription factor can repress the activity of the NLRP1 and NLRP3 inflammasome thereby subduing IL-1β production [144]. For the AIM2 inflammasome, *Irf3* deficient mouse macrophages, which are unable to secrete type I interferons, have impaired AIM2 activation in response to *Francisella tularensis* infection

indicating that an intact type I interferon response is required for AIM2 activation [125]. Interestingly, activation of the AIM2 inflammasome in response to mouse cytomegalovirus does not require an intact type I interferon response [127]. The mechanisms pertaining to the selective requirement of type I interferons for the clearance of certain infections remain unclear.

Evidence suggests that cells of the adaptive immune response can also dampen inflammasome activation. In mouse macrophages and dendritic cells, effector CD4+ T cells and memory T cells suppress activation of the NLRP1 and NLRP3 inflammasomes. For the NLRP3 inflammasome the inhibitory effect requires the cell-to cell contact and could be mimicked by macrophage stimulation with members of the TNF family such as, CD40L, OX40L and RANKL. Interestingly, the negative feedback loop exerted by T cells is only evident for the NLRP1 and NLRP3 inflammasome and was absent for the NLRC4 inflammasome [145].

1.8.5 REGULATION FROM INFLAMMASOME COMPONENTS

Inflammasomes components can themselves indirectly impact on inflammasome formation and IL-1β release. For example, NLRP12 acts as a negative regulator of the NF-κB pathway through its interaction and regulation of NIK and TRAF3, and dysregulation of NF-κB is associated with colonic inflammation and cancer [146]. NLRP10 interacts with ASC, even though it lacks a ligand sensing LRR, and is thought to negatively regulate the inflammasome by sequestering ASC [147, 148]. The ASC adaptor protein, in addition to the full length ASC also exists as three novel isoforms, ASC-b, ASC-c and ASC-d. ASC-c exerted an inhibitory effect on NLRP3 inflammasome formation by only colocalise with caspase-1 and not

NLRP3. ASC-d failed to colocalise with either caspase-1 or NLRP3 suggesting an undetermined function for this isoform [67].

Emerging evidence indicates NLRP7 is able to regulate inflammasomes, however conflict reports argue the nature of the negative regulation. Reconstitution experiments in HEK293 cells have shown that NLRP7 inhibits NLRP3 and caspase-1 mediated release of IL-1β and co-immunoprecipitation studies indicated NLRP7 directly interacts with procaspase-1 and proIL-1β [149]. While other work focusing on NLRP7 overexpression and gene specific mutations have indicated that NLRP7 inhibits NF-κB activation by an unknown mechanism or inhibits release of IL-1β [150]. Positive regulation is affirmed by the formation of the NLRP7 inflammasome in response to microbial acylated lipopeptides [131].

1.9 DISEASE ASSOCIATED INFLAMMASOME RELATED VARIANTS

With regard to IBD, GWAS and met-analysis have identified 240 loci associated with disease development [21], however none of these loci involve inflammasome forming genes.

Several inflammatory and autoimmune diseases are linked to inflammasome variants and population studies have indicate the potential involvement of inflammasome loci in the development of IBD.

The hereditary periodic fever syndromes known Cryopyrin-associated periodic syndromes (CAPS) are associated with the gain of function in NLRP3 which causes continuous IL-1β secretion in the absence of an antagonist. CAPS are a family of autosomal dominant diseases including, familial cold auto-inflammatory syndrome (FCAS), Muckle-Wells syndrome, (MWS), and chronic infantile neurological, cutaneous and articular, (CINCA) syndrome. The clinical

severity of CAPs varies but in general all syndromes are characterised by recurrent fever, urticarial-like rashes and systemic inflammation [151]. More than 90 disease associated genetic variants of the *NLRP3* gene have been identified for CAPS, the majority are autosomal dominant missense point mutations, located in exon 3, which encodes the NACHT domain [152]. Remarkable clinical outcomes for CAPS patients have been achieved by the administration of IL-1 blocking agents such as rilonacept (Arcalyst, Regeneron, Tarrytown, NY, USA), canakinumab (Ilaris, Novartis, Basel, Switzerland) and anakinra (Kineret, Amgen, Thousand Oaks, CA, USA) [151].

The potential involvement of NLRP3 in the pathogenesis of IBD has prompted several population studies to examine in depth the genetic region of NLRP3. Contributing to CD susceptibility in individuals of European descent is a set of SNPs (rs4353135, rs4266924, rs55646866, rs6672995, rs107635144 and rs10733133) located in a predicted regulatory region on chromosome 1q44, downstream of *NLRP3*. The risk allele rs4353135 was associated with a decrease in *NLRP3* expression while rs6672995 was associated with hypoproduction of IL-1β [153]. A subsequent study in the UK showed no significant association on single locus, subphenotype or haplotype analysis for the above six SNPs with CD [154]. Similarly, in a Korean cohort, none of the four NLRP3 SNPs in this study were associated with CD or UC. However female UC patients homozygous for the rs2043211 SNP encoding the Cys10X in the CARD8 gene, which is the procaspase-1 recruiting domain of ASC, were significantly associated with high serum IL-1β levels [155]. Similar sex difference have been shown in a Swedish cohort where the genetic susceptibility of CD in men was associated with combined polymorphisms in CARD8 and NLRP3 [156]. Adding to this is the gain of function

SNP (Q705K) within the NLRP3 gene which has been associated with increased mortality in advanced colorectal cancer patients [157].

Given the inconsistencies in population based studies and suggested influence on disease progression further studies are warranted to determine the role NLRP3 variants play in IBD susceptibility and disease phenotypes.

SNPs in the promoter and coding region of *NLRP1* are associated with a high risk of developing a variety of diseases such as, generalised vitiligo, vitiligo autoimmune diseases, Addison's disease, type I diabetes, lupus, rheumatoid arthritis, autoimmune thyroid disease and Alzheimer disease [158-161]. Generalised vitiligo has shown strong association to genetic variations in *NLRP1* with individual carrying two independent risk signals from SNPs at rs6502867, rs2670660 and rs8182352 at higher odds ratio (4.2) than individuals carrying a high risk allele from only one signal [162]. More recently, SNPs in coding regions of NLRP1 (rs11621270) and CARD8 (rs2043211) were found to be associated with death and poor disease outcome in bacterial meningitis patients [163].

Functional studies have suggested multiple roles for CARD8 including, the negative regulation of NF-$_{K}$B,[75] regulation of apoptosis through the inhibition of caspase-1, caspase-8 and caspase-9 [74], and lastly as a physical component of the inflammasome complex [48]. CARD8 is expressed in the gastrointestinal epithelium and lies beneath a GWAS identified peak of linkage for CD on chromosome 19q. The rs2043211 SNP changes a cysteine residue to a premature stop codon at codon 10 resulting in truncated CARD8 protein which has the potential to disrupt not only inflammasome formation but other inflammatory mechanisms. The association of the rs2042311 polymorphism to inflammatory

bowel disease has been demonstrated in several independent studies which suggests CARD8 is a potential candidate gene for IBD [155, 156, 164, 165].

Because of IL-18 ability to induce IFN-ɣ and mediate Th1 and Th2 immune responses much attention has focused on the genetic associations of IL-18 with chronic inflammatory disorders such as, asthma, coronary heart disease, Type I diabetes, multiple sclerosis, rheumatoid arthritis, juvenile idiopathic arthritis, graft-versus-host–disease and Crohn's disease [166]. While disease specific polymorphisms have been identified, the genetic data is largely contradictory and divergent associations are numerous reflecting significant genetic heterogeneity within groups.

In the intestine IL-18 is expressed in intestinal epithelial cells, and macrophage and dentritic cells of the lamina propria [167]. The human IL-18 gene maps to 11q22.2–q22.3 and consists of 6 exons and 5 introns spaning over 20.8 kb [168]. Initial DNA sequencing of the entire coding region of IL-18 failed to identify functional mutations however a novel SNP identified as TCA/TCC at codon 35 was significantly higher in CD patients than healthy controls and more remarkable in females than males [169]. In a Japanese cohort, DNA direct sequencing identified a higher frequency of a -137G/C promoter polymorphism in UC proctitis patients but no significant difference was detecting in CD patients [170]. However in a German cohort the -137 (G/C) polymorphism was not a strong risk factor for IBD [171]. More recently, using sequence-specific polymerase chain reaction methods the -137 (G/C) SNP and another IL-18 gene promoter SNP, -607 (C/A) were identified as being associated with the development of UC but not CD [172]. In addition, functional candidate-gene analysis has suggested a strong association of the IL18RAP rs917997 SNP to both CD and UC [173]. Similarly,

meta-analysis of IL-18 polymorphism and UC identified three polymorphisms, rs1946518, rs187238 and rs360718 that may contribute to susceptibility for UC among Asians and Africans [174]. Taken altogether, the lack of consistent genetic associations for IL-18 suggest promoter polymorphism may impact more on disease progression rather than susceptibility.

Disease association studies for NLRC4 and NAIP variants are limited. A rare haplotype in the CARD4/ NLRC4 gene has been reported to be more frequent in atopic dermatitis patients than in control patients [175]. A truncated NAIP protein has been linked to deregulation of motor neuron apoptosis which contributes to the autosomal recessive phenotype known as spinal muscular atrophies [176]. More recently, two *de novo* gain of function mutations in human NLRC4, encoding p.Val341Ala and p.Thr337Ser substitutions, situated in the NACHT protein domain, are associated with a distinct autoinflammatory disease that cosgregates in families [177, 178]. The syndrome presents with gastrointestinal complaints, fever and systemic inflammation with varying degrees of severity. The genetic defect, now termed NLRC4-macrophage activated syndrome (MAS), is thought to result in constitutive activation of the NLRC4 inflammasome in macrophages and elevated levels of IL-1β and IL-18 [178].

1.10 INFLAMMASOMES AND THEIR IMPACT ON THE INTESTINAL ENVIRONMENT

Mouse models that stimulate colitis, such as DSS, have provided an accessible framework for investigating the role of inflammasomes in diseases that affect the gastrointestinal tract. Differences in the experimental conditions used for colitis induction and pathogen infection have resulted in many discrepancies regarding the redundant or necessary role of individual inflammasome complexes in protecting against colitis [71].

Mice deficient in *Nlrp3, Nlrc4, Il-1β, Casp1/11* and *Asc*, when challenged by DSS, have all shown increased susceptibility to colitis, disease exacerbation, frequent mortality and increased tumor formation when compared to DSS challenged wild type mice, suggesting these components aid colitis protection [179-185]. Disease exacerbation has also been a feature of DSS challenged *Nlrp6* deficient mice [186]. Not reported in *Nlrc4* and *Nlrp3* deficient mice but associated with Nlrp6 deficiency is a reduction in the thickness of the mucus layer and the development of a transferable colitis forming microbiota dominated by TM7 and *Prevotellaceae* species (*Bacteroidetes* phyla). The reduction in mucus has been attributed to defects in mucin granule exocytosis and reduce autophagy mechanisms in goblet cells which suggests that, unlike NLRP3 and NLRC4, NLRP6 orchestrates downstream mechanisms involved in bacterial defences [186, 187]. NLRP6 is reported to influence the composition of the microbial ecology with *Nlrp6, Il18, Asc* and *Casp1* deficient mice developing a colitis forming microbiota dominated by TM7 and *Prevotellaceae* (*Bacteroidetes* phyla) species [186] however these results have been refuted by further work using microbial phylogenetic analyses of littermate-controlled experiments [188].

Alterations in the composition of the gut microbiota have been reported for IBD patients [189, 190]. In general, UC patients exhibit higher overall bacterial counts while in CD the bacterial counts are lower but associated with a higher proportion of unclassified *Bacteroidetes* spp. and a higher diversity of TM7 phylotyes. Increases in *Enterobacteriaceae,* adherent-invasive strains of *Escherichia coli* and *Ruminococcus gnavus* populations and a decrease in *Faecalibacterium* and *Roseburia* have been reported for ileal CD [191-193]. Interestingly, disease remission in UC induces microbial populations comparable to healthy patients while in CD the microbial population is reportedly not altered by disease remission, remaining constant in active and quiescent disease states [191].

1.11 AIMS AND HYPOTHESIS

Inflammasome complexes are core components of the innate immune response and are responsible for orchestrating multiple downstream pathways to aid pathogen elimination and tissue repair. Dysregulation of the inflammasome complex is hypothesised to be a contributing factor in the development of gastrointestinal diseases. Research examining the role of inflammasomes in human IBD are currently lacking and therefore the overall aim of this study was to examine the activity of inflammasomes in human IBD.

The specific aims of this research are;

1. To determine the mRNA expression of inflammasome components in colon biopsies procured from quiescent and active disease regions using qRT-PCR and RNA sequencing.
2. To examine the cellular localisation of inflammasome receptors and the IL-1β cytokine using immunohistochemistry and immunofluorescence confocal microscopy.
3. To investigate the effect of *NLRP6* induction on *MUC2* expression in colonic cell lines.

CHAPTER 2 MATERIAL AND METHODS

2.1 ETHICS

This research was conducted by the University of Tasmania, in collaboration with local Gastroenterologists, St Vincent's Private Hospital, Launceston General Hospital and Launceston Pathology. Human studies were approved by the University of Tasmania (ethics approval: H11930) and Calvary Health Care of Tasmania Clinical and Ethics committee (reference number: 03:09:12). Approval letters are provided in Appendix 1 and 2. All participant in this study provided informed written consent or parental consent and were aged between 15 and 80 years. Additional information relating to patient demographics, medication, co-morbidities, smoking and medical history was collected prior to the procedure and confirmed post operation with the treating clinician. Patient participation information, medical history and consent forms are provided in Appendix 3 and 4. To protect the privacy of participants, patient names and personal details were blinded from University of Tasmania researchers.

2.2 STUDY PARTICIPANTS AND BIOPSY COLLECTION

A total of 85 patients presenting for routine colonoscopy investigations between June 2012 and June 2014 were recruited for this study. IBD patients were excluded if they were experiencing co-existing irritable bowel syndrome (IBS) or non-IBD associated gastrointestinal bleeding at the time of colonoscopy. Control patients were excluded if they had a previous history of IBD or IBS.

Of the 85 participants, 30 UC patients and 15 CD patients had colonic biopsies taken from both inflamed mucosa and non-inflamed mucosa. Ten UC patients and

four CD patients were in remission at the time of colonoscopy and only had biopsies taken from non-inflamed mucosa. Four UC and two CD patients presented with colonic disease in its entirety and had biopsies taken from only inflamed mucosa. Twenty control patients had biopsies collected from healthy non-inflamed mucosa. Disease assessment and biopsy location was at the discretion of the treating Gastroenterologist. Biopsies for quantitative real-time polymerase chain reaction (qRT-PCR) analysis were stabilised with Allprotect (QIAGEN, Venlo, Netherlands) and frozen at -80^{0}C pending RNA isolation. Biopsies for immunohistochemistry and immunofluorescence confocal microscopy analysis were fixed with 10% v/v buffered formalin and paraffin embedded. Selection was based on patient pathology reports and only biopsies from the descending left colon with active disease features or those diagnosed as in IBD remission were chosen for microscopy analysis.

2.3 RNA ISOLATION

Total RNA was extracted from quiescent and active diseased colon biopsies using the RNeasy Plus Mini Kit (QIAGEN, Venlo, Netherlands) as per manufacturer's instructions. Briefly, the Allprotect stabilised tissue was disrupted in 350µL of RLT lysing buffer and the tissue was homogenised using a 25-gauge needle attached to a 1ml syringe. The homogenised lysate was transferred into an RNeasy mini spin column and treated at room temperature with a series of buffers to wash the membrane bound RNA. The RNA was then eluted with 20µL of nuclease-free water. RNA quality and concentration were determined using the Experion automated electrophoresis system (BIO-RAD, Hercules, CA, USA).

Samples with a calculated RQI ≥ 8.0 were deemed suitable for qRT-PCR analysis or RNA sequencing.

2.4 TRANSCRIPTIONAL PROFILING WITH RNA-SEQUENCING

RNA samples for sequencing were transported on dry ice to:

Ramaciotti Centre for Genomics

Level 1, Biological Sciences Building (D26)

The University of New South Wales

NSW 2052

Telephone: +61 (2) 9385 1658

Facsimile: +61 (2) 9385 1277

http://www.ramaciotti.unsw.edu.au

At Ramaciotti, RNA quality, concentration and purity were confirmed using the Agilent 2100 Bioanalyzer system (Agilent, Santa Clara, CA, USA). Library preparation was performed using the SureSelect targeted RNA capture kit (Integrated Sciences, Sydney, Australia) and libraries were validated on the Illumina HiSeq 2000 Sequencer (v2 100bp, pair-end [PE] reads) (Illumina, San Diego, CA, USA) at a concentration of 80Mb. Base-calling was performed off-line using Galaxy by Dr Dale Kunde at the University of Tasmania.

2.5 COMPLEMENTARY DNA PREPARATION

RNA was reverse transcribed to cDNA using the iScript cDNA synthesis kit (BIO-RAD, Hercules, CA, USA) as per manufacturer's instructions. Briefly, the Experion RNA concentration results (ng/µl) determined the volume of RNA needed to produce 1µg of cDNA product. For each sample the RNA was combined with iScript Supermix, reverse transcriptase and nuclease free water to a final volume of 20µL. Samples were incubated at 25^0C for 5 minutes, 30 minutes at 42^0C followed by 5 minutes at 85^0C and then held at 4^0C. Samples were frozen at minus 80^0C pending qRT-PCR analysis.

2.6 GENE EXPRESSION ANALYSIS

qRT-PCR was performed on cDNA, at a concentration of 25ng/ml, using TaqMan Universal PCR Master mix (Applied Biosystems, Foster City, CA, USA), on-demand human gene specific primers (Applied Biosystems, Foster City, CA, USA) (Table 2-1) and the StepOne Plus PCR system (Applied Biosystems, Foster City, CA, USA). A 40 minute fast thermal cycling profile was used for gene expression analysis and included a 2 minute Uracil-N glycosylate (UNG) treatment step at 50^0C, a 20 second polymerase activation step at 95^0C, and 40 cycles of amplification (denaturation for 1 second at 95^0C, annealing and extension for 20 seconds at 60^0C). All samples were amplified simultaneously in a one run per designated primer/probe assay.

Table 2-1: Pre-designed Taqman primer/probes used for gene expression analysis

Gene symbol	Gene name	Catalogue identification
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	Hs02758991_g1
EEF2	Eukaryotic translation elongation factor 2	Hs00157330_m1
HPRT1	Hypoxanthine phosphoribosyltransferase 1	Hs02800695_m1
ACTB	beta-actin	Hs01060665_g1
IL-1β	Interleukin 1, beta	Hs01555410_m1
IL-18	Interleukin 18 (interferon-gamma-inducing-factor)	Hs01038788_m1
NLRP1	NLR family, pyrin domain containing 1	Hs00248187_m1
NLRP3	NLR family, pyrin domain containing 3	Hs00918082_m1
NLRP6	NLR family, pyrin domain containing 6	Hs00373246_m1
AIM2	Absent in melanoma 2	Hs00915710_m1
SLC7A10 (ASC)	Solute carrier family 7 (neutral amino acid transporter light chain, asc system), member 10	Hs00219811_m1
CASP1	Caspase-1, apoptosis related cysteine peptidase	Hs00354836_m1
NOD1	Nucleotide-binding oligomerisation domain containing 1	Hs00196075_m1
NOD2	Nucleotide-binding oligomeriszation domain containing 2	Hs00223394_m1
PPAR-γ	Peroxisome proliferator-activated receptor gamma	Hs01115513_m1

2.7 IMMUNOHISTOCHEMISTRY ANALYSIS

Immunohistochemistry was performed on 10% v/v formalin fixed, paraffin embedded human tissue. Sections were deparaffinised and subjected to 0.01 M citrate buffer (pH 6.0) antigen retrieval at 121^{0}C for 4 minutes in a decloaking chamber. Endogenous peroxidases were quenched by a 5 minute incubation in 10% H_2O_2 in methanol. Sections were washed 3 times for 3 minute with 0.1 mol/L Tris-buffered saline (TBS). Non-specific binding was blocked by a 20 minute incubation with Biocare background Sniper solution (BS966G, Biocare, Concord, CA21520, USA). Sections were again washed 3 times for 3 minute with TBS. Immunostaining was performed using specific antibodies against IL-1β (rabbit polyclonal, ab9722, Abcam, Cambridge, MA, USA, 1:300), CIAS1/NALP3 [nalpy3-b] (mouse monoclonal, ab17267, Abcam, Cambridge, MA, USA, 1:300), AIM2 (rabbit polyclonal, ab93015, Abcam, Cambridge, MA, USA, 1:500), NLRP6 (rabbit polyclonal, NBP2-31372, Novus Biological, Littleton, CO, USA, 1:200) and MUC2 (rabbit polyclonal, H:300: sc15334, Santa Cruz, Dallas, Texas, USA, 1:300), at room temperature for 1 hour. Sections were again washed 3 times for 3 minutes with TBS. Sections with the anti-CIAS1/NALP3 [nalpy3-b] antibody added were incubated with a MACH 1 mouse probe (UP537L10, Biocare, Concord, CA21520, USA) at room temperature for 15 minutes. All sections were incubated with a horseradish peroxide (HRP)-polymer (MRH53BL10, Biocare, Concord, CA21520, USA) for 30 minutes, washed 3 times for 3 minutes with TBS and stained with Betazoid DAB chromogen (BDB900B, Biocare, Concord, CA21520, USA) for 5 minutes. Sections were rinsed with distilled water, counterstained with hematoxylin, dehydrated in alcohol/xylene and mounted with distyrene, plasticiser and xylene (DPX) mounting media.

Slides were examined using an IX71 microscope (Olympus Australia, Melbourne, Australia) and images were captured using an attached DP21 microscope camera (Olympus Australia, Melbourne, Australia).

2.8 IMMUNOFLUORESCENCE CONFOCAL MICROSCOPY

Immunofluorescence staining was performed on 10% v/v formalin fixed, paraffin embedded human tissue. Sections were deparaffinised and subjected to 0.01 M citrate buffer (pH 6.0) antigen retrieval at 121^{0}C for 4 minutes in a decloaking chamber. Non-specific binding was blocked by a 1 hour incubation in blocking buffer [0.1 mol/L phosphate-buffered saline (PBS) / 5% normal goat serum (ab7481, Abcam, Cambridge, MA, USA) / 0.05% Tween-20] at room temperature in the dark.

Immunofluorescence staining was performed using specific antibodies against IL-1β, CIAS1/NALP3 [nalpy3-b], NALP3 [nalpy3-α], NLRP6, AIM2, MUC2 and E-cadherin (Table 2-2) at room temperature in the dark for 1 hour, or alternatively overnight at 4^{0}C. Primary antibody was washed 3 times for 5 minutes with 0.1 mol/L PBS. Sections were incubated for 1 hour in the dark with one or more of the following fluorochrome conjugated-secondary antibodies diluted 1:500 in [0.1 mol/L PBS / 5% normal goat serum / 0.05% Tween-20]; Goat Anti-Mouse (H+L) F(ab')$_2$ Alexa Fluor®555 Conjugate (#4409, Cell Signalling Technology, Danvers, MA, USA), Goat Anti-Rabbit (H+L) F(ab')$_2$ Alexa Fluor®555 Conjugate (#4413, Cell Signalling Technology, Danvers, MA, USA), Goat Anti-Mouse (H+L) F(ab')$_2$ Alexa Fluor®647 Conjugate (#4410, Cell Signalling Technology, Danvers, MA, USA), Goat Anti-Rabbit (H+L) F(ab')$_2$ Alexa Fluor®647 Conjugate (#4414, Cell Signalling Technology, Danvers, MA, USA). Section were then washes 3 times

for 5 minutes with PBS. Section were incubated for 10 minutes with 4' ,6 diamidino-2-phenylindole Dihydrochloride (DAPI, (#D1306, ThermoFisher Scientific, Waltham, MA, USA) diluted in PBS, washes 3 times for 5 minutes with PBS, mounted with ProLong® Gold Antifade (P36930, ThermoFisher Scientific, Waltham, MA, USA) and allowed to harden at room temperature for 24 hours before imaging. Slides were examined using an FV1200 Laser Scanning Confocal Inverted Microscope (Olympus Australia, Melbourne, Australia). Images were captured and analysed using a combination of the Olympus IX83 software and the off-line FLUOVIEW software. It is important to note that immunofluorescence staining on paraffin embedded sections produces images with slightly less sensitivity when compared to immunohistochemistry and this can be attributed to the inability of immunofluorescence imagery to distinguish between cell borders and background tissue.

Table 2-2: Primary antibodies used for immunofluorescence confocal microscopy

Primary Antibody	Source	Manufacturer	Product Number	Dilution
IL-1β	Rabbit polyclonal	Abcam, Cambridge, MA, USA	ab9722	1:100
CIAS1/NALP3 [nalpy3-b]	Mouse monoclonal	Abcam, Cambridge, MA, USA	ab17267	1:100
NALP3 [nalpy3-α]	Mouse monoclonal	Abcam, Cambridge, MA, USA	ab16097	1:100
NLRP6	Rabbit polyclonal	Novus Biological, Littleton, CO, USA	NBP2-31372	1:200
AIM2	Rabbit polyclonal	Abcam, Cambridge, MA, USA	ab93015	1:100
MUC2	Rabbit polyclonal	Santa Cruz, Dallas, Texas, USA	H-300:sc-15334	1:200
MUC2	Mouse monoclonal	Santa Cruz, Dallas, Texas, USA	F-2:sc-515032	1:200
E-cadherin	Mouse monoclonal	Dako North America, Carpinteria, CA, USA	NCH-38; M3612	1:50

2.9 CELL CULTURE EXPERIMENTS

The human colorectal adenocarcinoma cell line, LS174T was obtained from Sigma-Aldrich, Corp. St Louis, MO, USA and cultured in RPMI Medium 1640 (Gibco, Gaithersburg, MD, USA) supplemented with 10% foetal bovine serum (Life Technologies, Carlsbad, CA, USA), glutaMAX™ (Life Technologies, Carlsbad, CA, USA), and 1% penicillin/streptomycin (Cellgro, Corning Life Sciences, Tewksbury, MA).

The human colon adenocarcinoma cell line, HT29 was obtained from Sigma-Aldrich, Corp. St Louis, MO, USA and cultured in McCoy's 5A Medium supplemented with 10% foetal bovine serum (Life Technologies, Carlsbad, CA, USA), and 1% penicillin/streptomycin (Cellgro, Corning Life Sciences, Tewksbury, MA).

Both cell lines were maintained in a 37^0C incubator with 5% CO_2 and a relative humidity of 95%. Cells between passage 6-10 were washed with Hanks balanced salt solution 1x (HBBS) (Life Technologies, Carlsbad, CA, USA), dissociated using TrypLE Express (Life Technologies, Carlsbad, CA, USA), and seeded at $1.2x10^5/cm^2$ one day prior to a 6 hour incubation with varying concentration of rosiglitazone (chemical abstract service number: 122320-73-4, Sigma-Aldrich Corp. St Louis, MO, USA). Cells were washed with HBBS, harvested and RNA was extracted using the RNeasy Plus Mini Kit (QIAGEN, Venlo, Netherlands) as per manufacturer's instructions. Briefly, cells were disrupted with 350µL of RLT lysing buffer and the homogenised lysate was transferred into an RNeasy mini spin column where it was treated with a series of buffers to wash the membrane bound RNA. The RNA was then eluted with 50µL of nuclease-free water. RNA

quality and concentration was checked using the Experion automated electrophoresis system (BIO-RAD, Hercules, CA, USA).

RNA was reverse transcribed to cDNA using the iScript cDNA synthesis kit (BIO-RAD, Hercules, CA, USA) as per manufacturer's instructions. Briefly, the RNA was combined with iScript Supermix, reverse transcriptase and nuclease free water to a final volume of 20µL. Samples were incubated at 25^0C for 5 minutes, 30 minutes at 42^0C followed by 5 minutes at 85^0C and then held at 4^0C. Samples were frozen at minus 80^0C pending qRT-PCR analysis.

qRT-PCR was performed using TaqMan Universal PCR Master mix (Applied Biosystems, Foster City, CA, USA), on-demand human gene specific primers (Applied Biosystems, Foster City, CA, USA) (Table 2-1) and the StepOne Plus PCR system (Applied Biosystems, Foster City, CA, USA).

A 40 minute fast thermal cycling profile was used for gene expression analysis and included a 2 minute Uracil-N glycosylate (UNG) treatment step at 50^0C, a 20 second polymerase activation step at 95^0C, and 40 cycles of amplification (denaturation for 1 second at 95^0C, annealing and extension for 20 seconds at 60^0C). Experiments were performed in duplicate on 3 separate occasions.

2.10 DATA ANALYSIS

Analysis of qRT-PCR data was performed using a combination of Microsoft Excel and GraphPad Prism software (version 7, GraphPad Software Inc. CA. USA). Briefly, in Excel the average CT results generated from RT-PCR were grouped accordingly and the mean control group CT for the reference gene (Ref.G) and genes of interest (G.O.I) were calculated. Sample ΔCT and relative expressions were calculated using the following,

ΔCT G.O.I = (mean control CT - sample CT), and

Relative Expression (R.E) G.O.I = (Efficiency G.O.I ^ ΔCT G.O.I/Efficiency Ref.G ^ ΔCT Ref.G).

Gene expression data are presented as scatterplots with horizontal lines indicating the median R.E and interquartile ranges and group difference were tested using paired t tests of log (10) transformed data. Cell line gene expression data are presented as mean ± S.E.M and group differences were assessed using one-way analysis of variance (ANOVA).

Quantitative analysis of immunohistochemistry data was performed using the FIJI version (Dec 2009) of ImageJ software (http://imagej.nih.gov/ij/downloads.html, Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA), Microsoft Excel and GraphPad Prism (version 7, GraphPad Software Inc. CA. USA). Briefly, the mean intensity of the DAB signal was measured in FIJI, optical density was calculated in Excel using log (maximum intensity/mean intensity), where the maximum intensity of an 8-bit image = 255. Images containing partial tissue were excluded from analysis because of their potential to bias results by reducing the average optical density. Group differences and statistical significance were evaluated using one-way

analysis of variance (ANOVA) followed by Dunnett's multiple comparisons in GraphPad Prism 7. All data are presented as mean $\pm$ standard deviation (SD). Spatial intensity profiling was performed using FIJI and GraphPad Prism was used to graph intensity plots.

Colocalisation analysis was performed using the Coloc 2 plugin in the Fiji version (Dec 2009) of ImageJ software (http://imagej.nih.gov/ij/downloads.html, Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA). To ensure consistency, measurements were only performed on biological relevant regions of interest (ROIs) contained within 400X double stained, background corrected, confocal images. Briefly in Coloc 2, the Costers method was used to determine a threshold point where the two channels of interest produced a Pearson's correlation coefficient of zero. Above this auto threshold point all pixels have a correlation greater than zero, while below all pixels have none or anti correlated intensities. Using Manders correlation coefficients (M1 and M2) the co-occurrence of the two channels is described and increases from 0 to 1 with increasing colocalisation. Pearson's correlation coefficient (above the threshold) was used to describe the correlation of the intensity distribution between channels. Quantitative analysis of Manders and Pearson's correlation coefficients was performed on lamina propria ROIs within 400X double stained confocal images (n=20). 2D histograms generated in Coloc 2 provided a visual inspection of the overall relationship of channel intensities for homologous pixels in a selected ROI. Where biological relevant ROIs were void of tissue (for example within colonic crypts), spatial intensity profiling was used to assess colocalisation of the primary antibodies.

Colocalisation data are presented as mean $\pm$ standard deviation. Statistical significance of Pearson's correlation for the NLRP3/IL-1β double staining was evaluated in GraphPad Prism (version 7, GraphPad Software Inc. CA. USA), using one-way analysis of variance (ANOVA), followed by Tukey's multiple comparisons test.

In all presented data the significance threshold was set at $p<0.05$ and only significant data is indicated.

CHAPTER 3 GENE EXPRESSION OF INFLAMMASOME COMPONENTS IN THE INFLAMMATORY BOWEL DISEASES

3.1 INTRODUCTION

The gastrointestinal tract is occupied by a wide repertoire of pathogenic and symbiotic microorganisms, which interact to shape innate and adaptive immune responses. Homeostasis is maintained by an array of germline-encoded pattern recognition receptors which detect PAMPs or DAMPs released from pathogen insult or cellular stress. Activation of pattern recognition receptors, such as the cytosolic NLRs, CLRs and membrane bound TLRs initiates the signalling pathways responsible for downstream pathogen elimination or tissue repair [49-51].

In response to DAMPs and PAMPs several members of the NLR receptor family, NLRP1, NLRP2, NLRP3, NLRC4, NLRP6, NLRP7 and NLRP12, and two members of the pyrin and HIN domain containing (PYHIN) family, absent in melanoma 2 (AIM2) and the interferon inducible protein (IFI16), also form the structural backbone of the innate immune complex known as the inflammasome [48, 68, 194]. The inflammasome consists of a central platform comprising oligomerised receptor molecules, a caspase and often an adaptor protein known as, apoptosis-associated speck-like protein containing a CARD (ASC). Inflammasomes regulate the maturation of the potent proinflammatory cytokines IL-1β and IL-18 through the activation of caspase-1.

Formation of the inflammasome complex is generally regarded as a two-step process [86]. Step one, is the priming step and involves receptor signalling to induce NF-$_{K}$B transcription of inflammasome components, proIL-1β and proIL-

18. Step two, involves a another signal which enhances inflammasome oligomerisation, caspase-1 activation, cytokine cleavage, maturation and cellular release [97].

For many of the inflammasome forming receptors the activating ligands remains unknown. The NLRP1 inflammasome is activated by bacterial MDP and lethal toxin (LeTx) produced by *Bacillus anthracis* [84, 195]. The NLRP3 inflammasome activates upon exposure to a wide range of whole pathogens, environmental irritants and structurally diverse DAMPs and PAMPs [48, 49, 86]. NLRC4 is activated by two critical components of pathogenic bacteria, a sequence motif found in the basal rod components of the type III (T3SS) and IV (T4SS) bacterial secretion systems, and a similar sequence motif found in flagellin, which is a component of their flagellum apparatus [114, 115]. Both the cytosolic AIM2 receptor and the nuclear IFI16 receptor directly bind their activating ligand double stranded DNA (dsDNA) via the C terminal HIN200 domain [120-122]. The mechanisms that enable AIM2 and IFI16 to respond to viral, bacterial, mammalian and synthetic dsDNA while remaining unresponsive to self DNA are still unclear [68, 124]. In human macrophages, NLRP7 forms an ASC-dependent inflammasome in response to microbial acylated lipopeptides [131]. The signals that activate the NLRP6 and NLRP12 inflammasomes are yet to be determined [128].

Important to innate immune defences are NOD1 and NOD2, which belong to the NLR family of cytosolic receptors. NOD1 senses intracellular gamma-D-glutamyl-meso-diaminopimelic acid (iE-DAP) found mainly in Gram-negative bacteria and only on some select Gram-positive bacteria, such as *Listeria* and *Bacillus* species [196-198]. NOD2 has been shown to recognise MDP, a peptidoglycan fragment,

found in most Gram-negative and Gram-positive bacteria, and single-stranded RNA of viruses [48, 195, 199-202].

The two main inflammasome producing cytokines, IL-1β and IL-18 are structurally similar but differ with respect to biological function. IL-1β is a multifunctional cytokine and is involved in generating local and systemic responses to injury, infection and inflammation. Clinically, IL-1β has the ability to evoke fever and hypotension [203], and control certain central nervous system functions such as sleep, appetite and pain [70]. Locally, IL-1β can induce cytokine production; stimulate T cell proliferation, and direct neutrophils to the site of injury or infection [50, 204, 205].

IL-18, is known mainly for its ability to induce IFN-ɣ, however other functions include, promoting synthesis of proinflammatory mediators [206], inducing endothelial cell migration and cell regeneration [207], and stimulating epithelial cell barrier function by the upregulation of adhesion molecules [132, 208]. The expression of proIL-1β is induced by the NF-κB transcription factor, and only in response to stimulation from TLRs or CLRs, whereas proIL-18 is constitutively expressed and its expression is increased after TLR receptor activation [55, 56].

PPARs are ligand-activated transcription factors belonging to a superfamily of nuclear receptors. PPARs are encoded by distinct genes located on different chromosomes and consist of three main members, PPAR-α, PPAR-γ and PPAR-β/δ. PPAR-γ has been linked to the regulation of intestinal inflammation and is highly expressed in the colon [209]. PPAR-γ operates as functional heterodimer with the retinoid X receptor (RXR) and together are bound to the promoter region of target genes by peroxisome proliferator response elements (PPREs). Upon ligand sensing of endogenous metabolites, dietary compounds or synthetic

drugs [210] the PPAR-γ/RXR heterodimer undergoes a conformational change that results in the transcriptional activation of target genes [211]. The use of transcription mapping has revealed potential PPAR-γ/RXR binding sites in regions upstream of the NLRP6 promotor, which are conserved across human, rat and mouse suggesting possible transcription control of NLRP6 by PPAR-γ [212].

Aberrant signalling of the inflammasome complex is hypothesised to be a key initiating event in the development of ulcerative colitis and Crohn's disease. The aim of this study was therefore to investigate the mRNA expression of inflammasome components in quiescent and active UC and CD using qRT-PCR and RNA Sequencing.

3.2 RESULTS

3.2.1 STUDY PARTICIPANT DEMOGRAPHICS

IBD patients comprised 65 out of the total 85 participants, and of these 35 were females and 30 were males. The mean age of IBD patients providing paired biopsies was slightly younger for the ileal (41 ± 16) and colonic (46 ± 15) CD group than the UC group (50 ± 18). Within the UC categories, patients presenting with colonic disease in its entirety were in general younger than the UC patients in remission or those with limited disease (Table 3-1). While in CD, the young age of remission patients could possibly be a reflection of close monitoring of newly diagnosed patients.

3.2.2 CLINICAL CHARACTERISTICS OF STUDY PARTICIPANTS

Forty-four UC patients participated in this study. At the time of biopsy collection, 27 were taking prescription medication to control their disease while 17 patients were untreated (Table 3-2). Eighty one percent (22/27) of treated UC patients were prescribed 5-aminosalicylates and 50% (11/22) of those taking 5-aminosalicylates were also prescribed corticosteroids. Approximately 55% of UC participants had coexisting medical conditions requiring prescription medication, 16% (7/44) experienced depression/anxiety, 11% had arthritis (5/44) and similarly 11% (5/44) had asthma. Reflux was a common complaint requiring prescription medication but this was thought to be a side effect of drug therapy or a reflection of lifestyle factors.

Twenty-one CD patients participated in this study, of these 76% (16/21) were being treated and 5 patients (24%) were not being treated at the time of biopsy collection (Table 3-3). While 5-aminosalicylates (29%, 6/21) and corticosteroids

(10%, 2/21) were often prescribed for CD, a larger proportion of CD patients were being treated with biologic agents (19%, 4/21) and immunomodulators (29%, 6/21). Forty-three percent (9/21) of CD participants experienced concurrent medical conditions with depression and anxiety disorders (24%, 5/21) being more frequent. Interestingly, there was a higher proportion of smokers in the CD group (24%, 5/21) when compared to the UC group (5%, 2/44).

Table 3-1: Demographic details of study participants

Patient category	Number of patients	Age (years)	Gender	Disease duration
Control patients	20	57 ± 14	11F, 9M	-
Paired UC*	30	50 ± 18	16F, 14M	11 ± 12
Quiescent UC only	10	49 ± 11	6F, 4M	15 ± 11
Active UC only	4	39 ± 21	2F, 2M	11 ± 13
Paired ileal CD*	6	41 ± 16	2F, 4M	12 ± 8
Active ileal CD only	1	28	1M	15
Paired Colonic CD*	9	46 ± 15	4F, 5M	8 ± 4
Active colonic CD only	1	46	1F	11
Quiescent CD (includes ileal CD and colonic CD) only	4	20 ± 7	3F, 1M	9 ± 14

All data are shown as mean ± standard deviation. The age of the subject during the biopsy is shown in years. Gender is given as F, females and M, males. The disease duration in years at the time of biopsy is shown for each group as the mean value ± standard deviation. The average onset of disease can be calculated by subtracting the disease duration from the average age of the patient.

*Patients had biopsies taken from quiescent disease and active disease regions

Table 3-2: Clinical characteristics of ulcerative colitis patients

Variable	Patients with both active and quiescent disease	Patients in remission	Patients with active disease only
Patients, n	30	10	4
Smoker, n (%)	1 (3)	1 (10)	0
Disease extent*			
Proctitis only	7 (23)	-	-
Proctosigmoiditis	14 (47)	-	-
Left-sided colitis	8 (27)	-	-
Pancolitis	1 (3)	-	4 (100)
Current medication for UC			
Untreated	12 (40)	3 (30)	2 (50)
Aminosalicylates (5-ASA)	16 (53)	5 (50)	1 (25)
Corticosteroids	6 (20)	3 (30)	2 (50)
Immunomodulator therapy	4 (13)	-	-
Topical therapy	0	-	-
Biologic therapy	0	-	-
Antibiotics/probiotics/ vitamin supplements	6	1	-
Concurrent medical conditions			
none	14 (47)	4 (40)	2 (50)
Asthma	5	-	-
Depression/ anxiety	5	-	2
Diabetes	1	1	-
High blood pressure	4	1	1
High cholesterol	3	1	-
Arthritis	2	2	1
Thyroid condition	1	-	-
Reflux	4	1	-
Gout	1	1	-

Age and disease duration data are shown as mean value $\pm$ standard deviation. Other data indicates the number of patients. Group percentages are shown in brackets.

*Disease extent:

Proctitis: Disease only in the rectum

Proctosigmoiditis: Disease in the rectum and sigmoid colon

Left-sided colitis: Limited or distal colitis. Disease in left side of the colon.

Pancolitis: Disease in the entire colon.

Table 3-3: Clinical characteristics of Crohn's disease patients

Variable	Patients with both active and quiescent disease	Patients in remission	Patients with active disease only
Patients, n	15	4	2
Smoker, n (%)	4 (27)	1 (25)	0
Disease extent*			
Ileum only	4 (27)	-	1
Ileum and colon	2 (13)	-	-
Colon only	9 (60)	-	1
Extra-intestinal manifestations (eg ileocaecal/perianal/proctitis)	4 (27)	-	-
Previous surgical resection	4 (27)	1	-
Current Medication for CD			
Untreated	4 (27)	1 (25)	-
Aminosalicylates (5-ASA)	5 (33)	1	-
Corticosteroids	2 (13)	-	-
Immunomodulator therapy	6 (40)	1	1
Topical therapy	-	-	-
Biologic therapy	2 (13)	1	1
Antibiotics/probiotics/ vitamin supplements	-	-	-
Concurrent medical conditions			
none	9 (60)	3 (75)	0
Asthma	1	-	
Depression/anxiety	2	1	2
Heart disease	1	-	-
Arthritis	1	-	-
Reflux	1	-	-
Iron deficiency	1	-	-
Ankylosing spondylitis	1	-	-

Age and disease duration data are shown as mean value ± standard deviation. Other data indicates the number of patients. Group percentages are shown in brackets.

*Disease extent: Extra-intestinal manifestations (eg ileocaecal/perianal/ proctitis) included as a separate section but can co-exist with colonic and ileum manifestations.

3.2.3 EXPRESSION OF TARGETED INFLAMMASOME GENES IN IBD

Quantitative RT-PCR and RNA sequencing were used to determine the expression of inflammasome related genes in colonic biopsies from quiescent and active disease regions. Justification for the use of EEF2 as the housekeeping gene for normalisation is presented in Appendix 5.

Overall, the expression of the inflammasome forming receptors, *AIM2, NLRP1, NLRP3* and *NLRP6* were found to increase with disease activity in both UC and CD (Table 3-4). The appearance of two distinct *NLRP6* expression populations in the active CD population prompted comparison with disease phenotypes. In patients with disease involving the terminal ileum (Montreal classification; L1, terminal ileum involvement and L3, ileocolonic involvement) the median relative expression of *NLRP6* increased 131-fold ($p<0.001$) with active disease (Table 3-4). In contrast, a 3.9-fold ($p=0.03$) increase in *NLRP6* expression was observed in colonic CD patients (Montreal classification; L2, colonic disease). The expression of *NLRP6* in active ileal CD was notably increased when compared to active colonic disease ($p<0.001$) (Figure 3-1D).

Given the overrepresentation of transcription factor binding sites for PPAR-γ upstream of the NLRP6 transcription start site [212] the expression profile of *PPAR-γ* was examined for evidence of possible transcriptional regulation of *NLRP6*. The expression of *PPAR-γ* decreased with disease activity for both UC and CD with the least expression evident in active ileal CD (0.2-fold reduction, $p=0.002$) (Table 3-4, Figure 3-1K).

The expression of *AIM2* was higher in active ileal CD (16.4-fold, $p=0.009$) than active colonic CD (10.7-fold, $p=0.014$) but comparable to active UC (10.2-fold, $p<0.001$) (Figure 3-1A). While the increased expression of *NLRP1* and *NLRP3*

was unremarkable when examined across active disease phenotypes (Figures 3-1B and 3-1C).

Consistent with inflammasome activation the expression of *IL-1β*, *CASP1* and *ASC* increased with disease activity in both UC and ileal CD (Figures 3-1E, 3-1G and 3-1H). Interestingly, the expression of *IL-18* remained constant in UC (p=0.10), ileal (p=0.24) and colonic CD (p=0.22) despite the increase in disease activity and demonstrated inflammasome activation (Figure 3-1F). Upregulation of inflammasome forming components in ileal and colonic CD were confirmed by RNA-sequencing (Figures 3-2 and 3-3).

The expression of the NOD bacterial sensor, *NOD2* increased with disease activity for both UC ($p<0.001$) and CD (ileal, p=0.009; colonic, $p<0.001$) however *NOD1* only increased in UC ($p<0.001$) (Figures 3-1I and 3-1J).

3.2.4 CORRELATION OF INFLAMMASOME COMPONENTS IN ACTIVE DISEASE

NLRP3 showed the strongest correlation with *IL-1β* for both UC (Rs=0.78, $p<0.01$) and CD (Rs=0.96, $p<0.01$) (Figure 3-4). The correlation of *IL-1β* to *NLRP1* and *AIM2* was weaker in both UC (*NLRP1*, Rs=0.48, <0.01; *AIM2*, Rs=0.47, $p<0.01$) and CD (*NLRP1*, Rs=0.65, $p<0.01$; *AIM2*, Rs=0.75, $p<0.01$). Interestingly, there was no correlation between *NLRP6* and *IL-1β* for both UC (Rs=0.32, p=0.09) and CD (Rs=0.12, p=0.65). *IL-18* showed the strong correlation to *CASP1* in both UC (0.79, $p<0.01$) and CD (Rs=0.73, $p<0.01$). Noteworthy the correlation of *AIM2* to *NLRP3* was stronger in active CD (Rs=0.83, $p<0.01$) than active UC (Rs=0.65, $p<0.01$) (Table 3-5).

Table 3-4: Expression of targeted inflammasome related genes in IBD

Target Gene	Disease phenotype	Quiescent disease		Active disease		P
		Median R.E	IQR	Median R.E	IQR	
AIM2	Ileal CD^	0.9	0.6 – 4.0	16.4	4.9 – 57.9	0.009
	Colonic CD+	1.8	0.7 – 4.9	10.7	5.2 – 14.3	0.014
	UC#	1.0	0.7 – 2.3	10.2	7.1 – 14.8	<0.001
NLRP1	Ileal CD^	2.0	0.7 – 2.4	4.2	2.7 – 6.3	0.002
	Colonic CD+	1.0	0.6 – 3.0	5.6	3.3 – 6.4	0.031
	UC#	1.4	1.0 – 1.6	4.9	3.3 – 6.3	<0.001
NLRP3	Ileal CD^	1.2	0.8 – 1.8	5.6	2.5 – 16.3	0.009
	Colonic CD+	2.7	1.1 – 3.1	8.5	2.6 – 17.3	0.024
	UC#	1.5	1.0 – 2.3	3.7	2.5 - 5.7	<0.001
NLRP6	Ileal CD^	1.4	0.7 – 1.7	**131.0**	109.1 – 260.4	<0.001
	Colonic CD+	1.7	0.6 – 2.9	3.9	2.9 – 5.6	0.03
	UC#	1.3	0.9 – 2.1	1.8	1.2 – 3.0	0.01
IL-1β	Ileal CD^	3.5	1.7 – 4.8	83.8	16.1 – 265.1	0.002
	Colonic CD+	4.1	1.5 – 19.2	63.5	9.4 – 309.4	0.02
	UC#	2.2	0.8 – 5.9	28.1	13.2 – 78.7	<0.001
IL-18	Ileal CD^	1.7	0.8 – 4.1	1.2	0.6 – 2.5	0.24
	Colonic CD+	1.1	0.7 – 3.2	2.3	1.3 – 4.9	0.22
	UC#	1.0	0.6 – 1.8	1.7	0.8 – 3.4	0.10

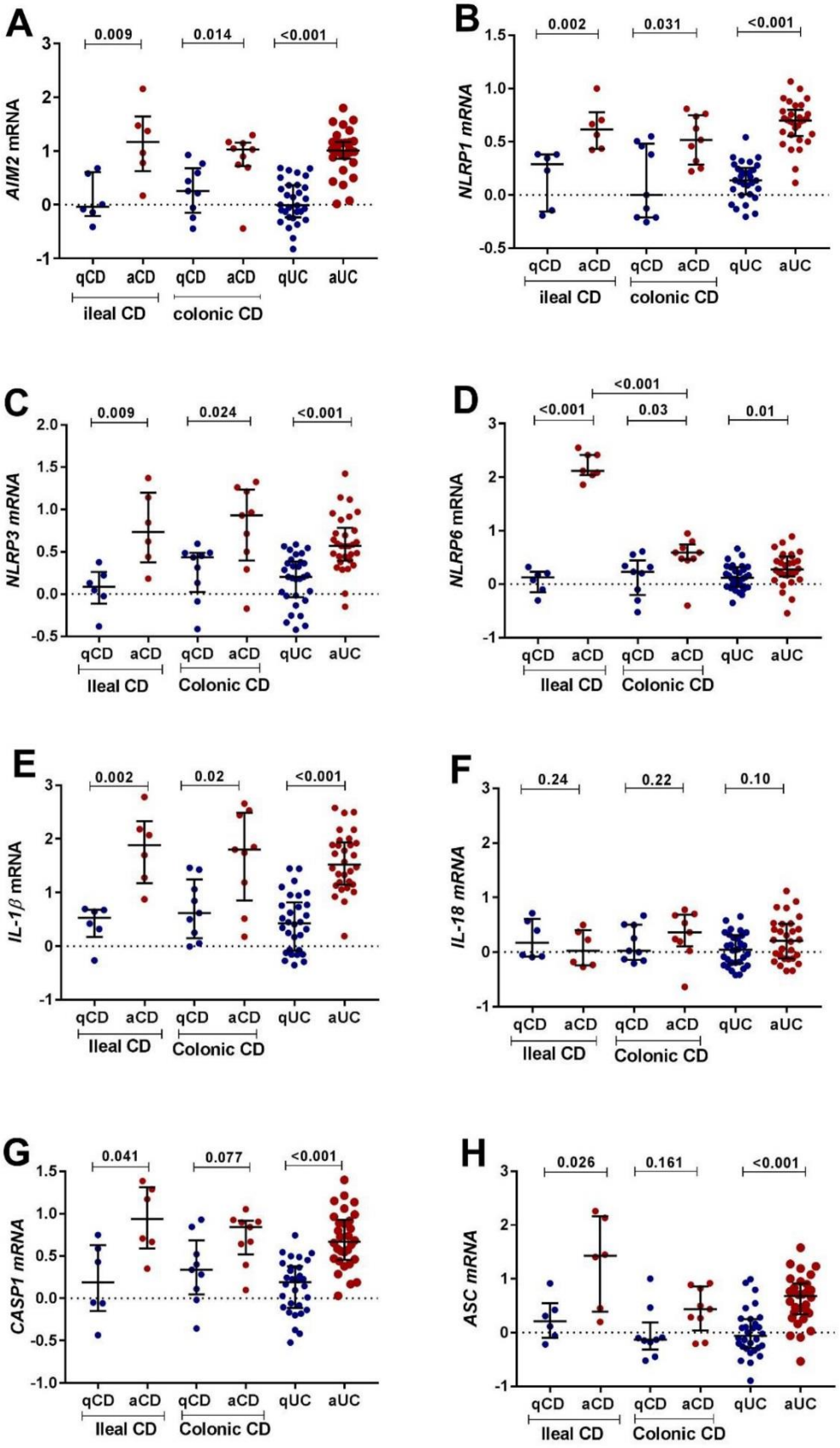
Target gene	Disease phenotype	Quiescent disease		Active disease		P
		Median R.E	IQR	Median R.E	IQR	
CASP1	Ileal CD^	1.8	0.7 – 4.3	10.0	4.0 – 20.7	0.041
	Colonic CD+	2.2	1.1 – 5.2	6.9	3.4 – 8.3	0.077
	UC#	1.3	0.8 – 2.3	5.1	2.9 – 9.3	<0.001
ASC	Ileal CD^	1.7	0.8 – 4.0	26.8	2.5 – 147.2	0.026
	Colonic CD+	0.7	0.5 – 1.9	2.7	1.3 – 7.2	0.161
	UC#	1.0	0.5 – 1.7	4.8	2.3 – 9.2	<0.001
NOD1	Ileal CD^	1.4	0.9 – 2.1	1.3	0.9 – 1.6	0.699
	Colonic CD+	1.1	0.7 – 1.8	1.5	1.3 – 1.7	0.190
	UC#	1.1	0.9 – 1.4	1.6	1.5 – 1.9	<0.001
NOD2	Ileal CD^	2.0	1.4 – 4.9	6.9	5.6 – 10.1	0.009
	Colonic CD+	2.5	1.8 – 3.4	5.8	3.9 – 19.3	<0.001
	UC#	1.5	0.9 – 2.1	8.0	4.6 – 10.4	<0.001
PPAR-γ	Ileal CD^	1.2	0.8 – 2.7	0.2	0.2 – 0.3	0.002
	Colonic CD+	0.9	0.6 – 1.4	0.6	0.3 – 0.9	0.113
	UC#	0.9	0.8 – 1.3	0.5	0.3 – 0.6	<0.001

Quantitative RT-PCR was used to determine the expression of inflammasome related genes in IBD. Median Relative Expression (R.E) and interquartile ranges (IQR) are relative to the control group and normalised to the housekeeping gene EEF2. Group differences were tested using Mann-Whitney test of log (10) transformed data. The significance threshold was $p<0.05$.

Column statistics for UC patients; quiescent disease n=40, active disease n=34; group difference for UC patients, n=30

^ Column statistics for ileal CD include 6 paired biopsy patients and one patient with active disease that was a known ileal CD patient

+ Column statistics for colonic CD = 9 patients


A
AIM2 mRNA
0.009
0.014
<0.001
qCD aCD qCD aCD qUC aUC
ileal CD colonic CD
B
NLRP1 mRNA
0.002
0.031
<0.001
qCD aCD qCD aCD qUC aUC
ileal CD colonic CD
C
NLRP3 mRNA
0.009
0.024
<0.001
qCD aCD qCD aCD qUC aUC
Ileal CD Colonic CD
D
NLRP6 mRNA
<0.001
<0.001
0.03
0.01
qCD aCD qCD aCD qUC aUC
Ileal CD Colonic CD
E
IL-1β mRNA
0.002
0.02
<0.001
qCD aCD qCD aCD qUC aUC
Ileal CD Colonic CD
F
IL-18 mRNA
0.24
0.22
0.10
qCD aCD qCD aCD qUC aUC
Ileal CD Colonic CD
G
CASP1 mRNA
0.041
0.077
<0.001
qCD aCD qCD aCD qUC aUC
Ileal CD Colonic CD
H
ASC mRNA
0.026
0.161
<0.001
qCD aCD qCD aCD qUC aUC
Ileal CD Colonic CD

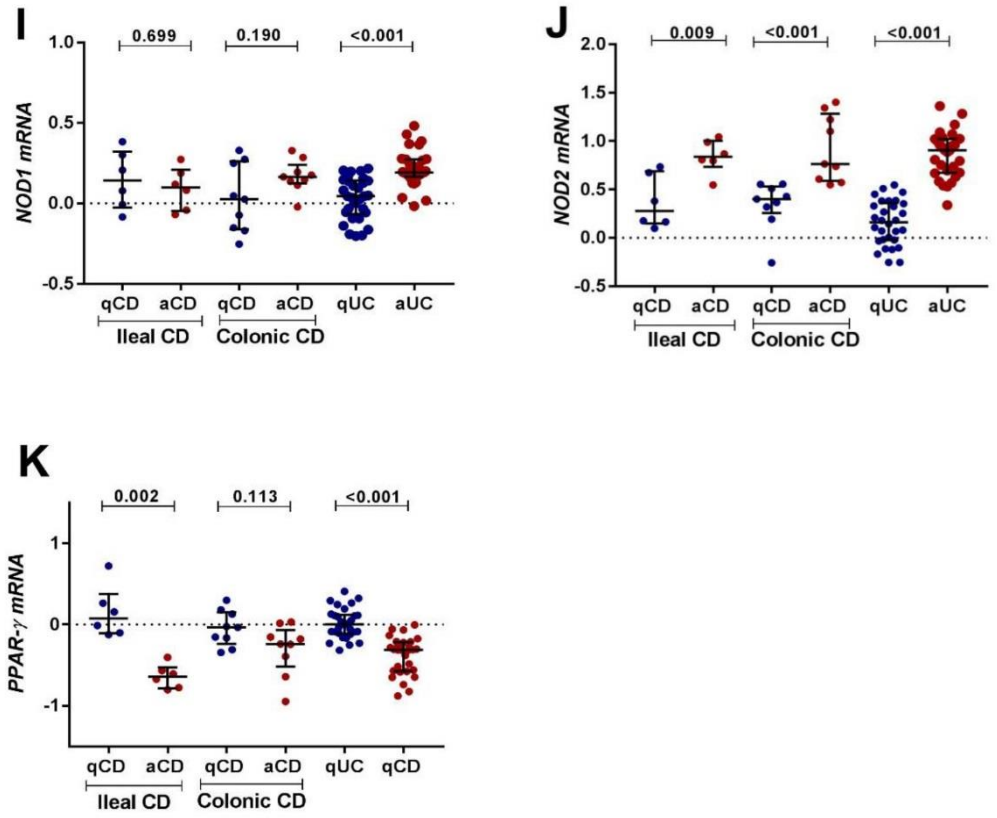


Figure 3-1: Gene expression of inflammasome related genes in paired biopsies from quiescent CD (qCD), active CD (aCD), quiescent UC (qUC) and active UC (aUC).

The mRNA expression of **A)** *AIM2*, **B)** *NLRP1*, **C)** *NLRP3*, **D)** *NLRP6*, **E)** *IL-1β*, **F)** *IL-18*, **G)** *CASP1*, **H**) *ASC*, **I)** *NOD1*, **J)** *NOD2*, **K)** *PPAR-γ* was compared to a healthy control group and normalised to the housekeeping gene, *EEF2*. Individual patient results are shown as dots. Horizontal lines indicate the median relative expression (R.E) and error bars represent the interquartile ranges. Group differences were tested using Mann-Whitney test of log (10) transformed data. The significance threshold was $p<0.05$.

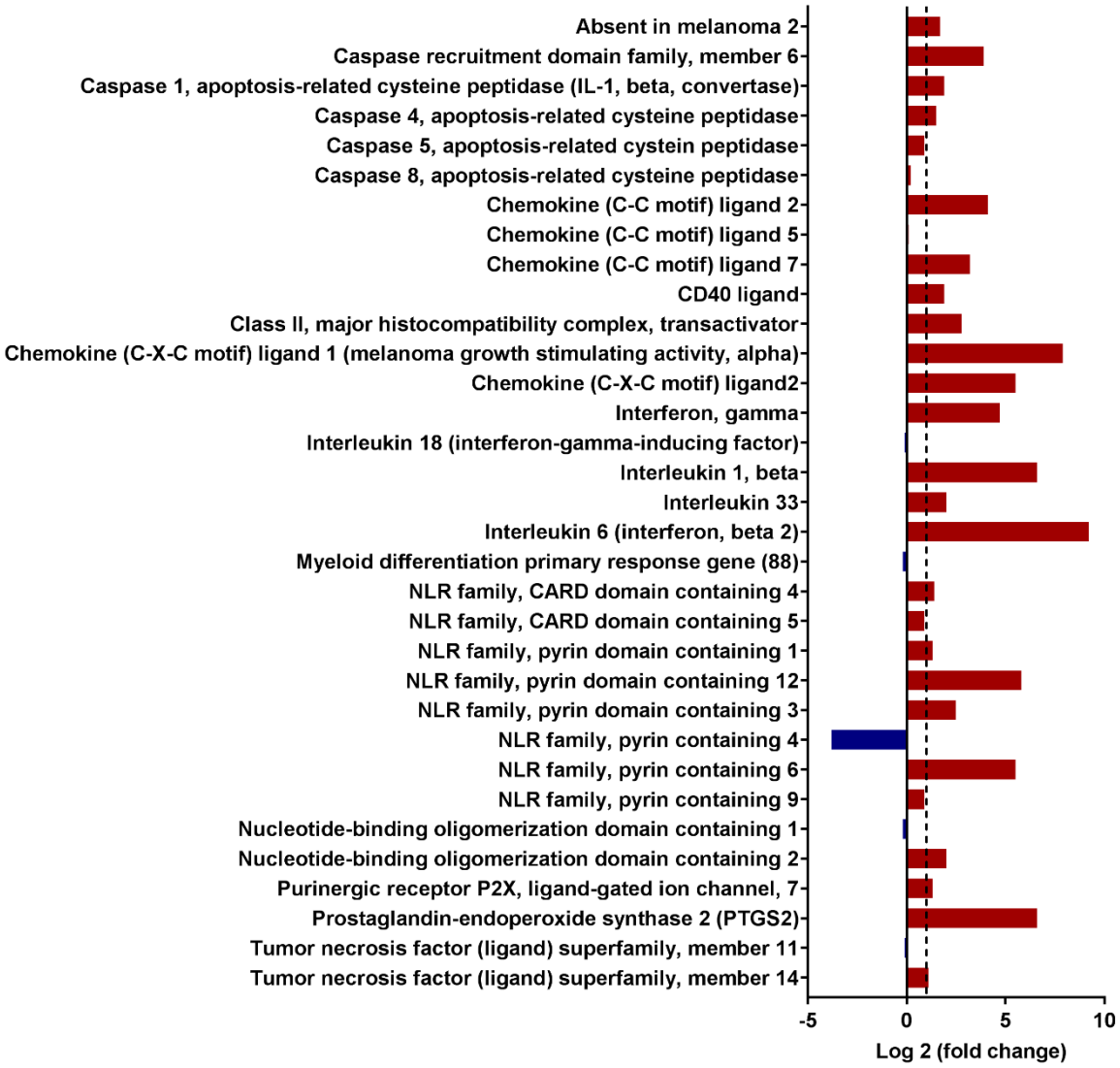


Figure 3-2: Targeted RNA-sequencing analysis of ileal CD colon biopsies during active disease.

Gene expression results (n=4) are expressed as Log 2 (fold change), normalised to housekeeping genes and relative to a normal control group (n=4) which is indicated by the dotted vertical line. Upregulated genes are shown as red, downregulated genes are shown as blue. The significance threshold was $p<0.05$.

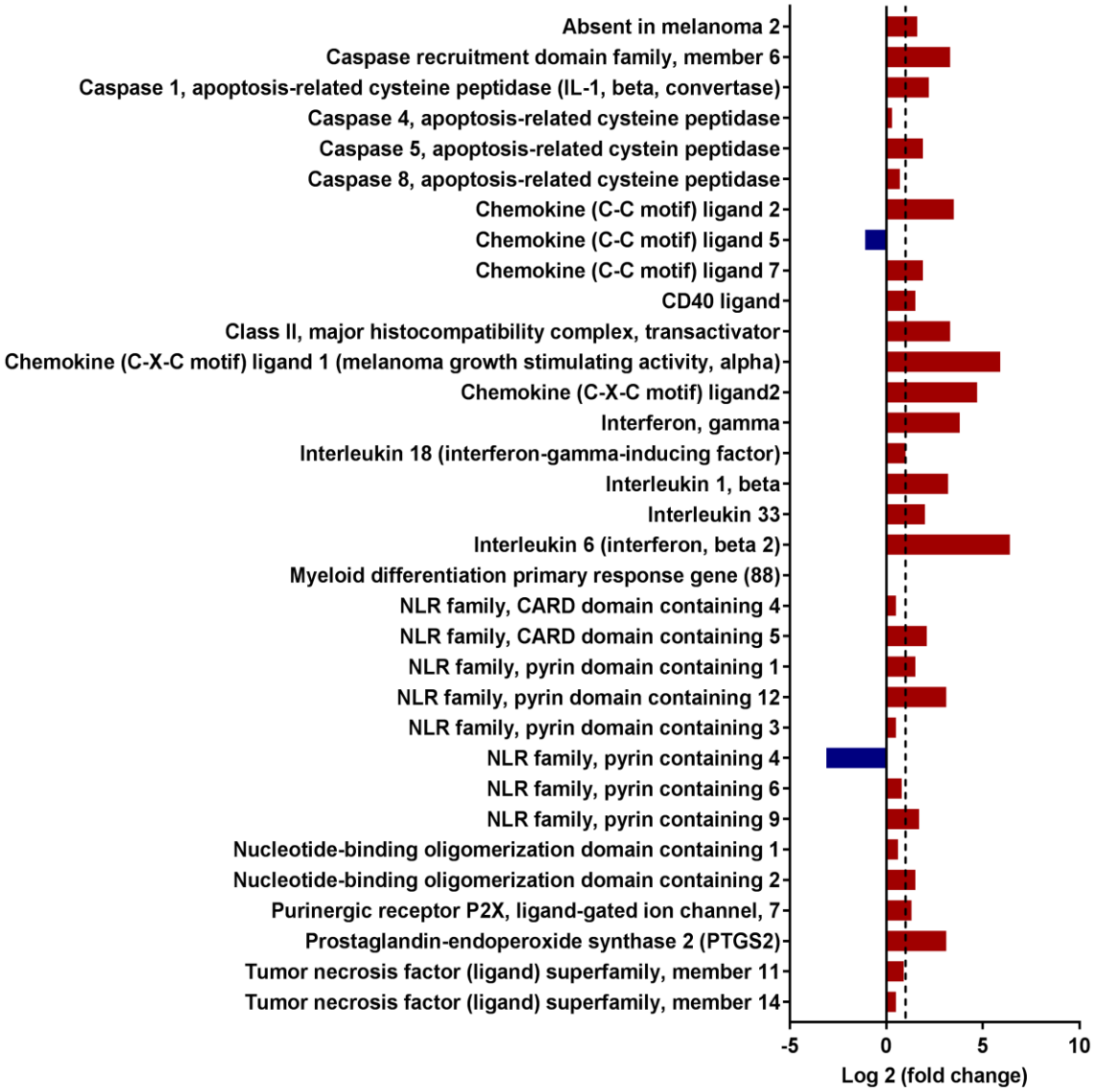


Figure 3-3: Targeted RNA-sequencing analysis of colonic CD colon biopsies during active disease.

Gene expression results (n=4) are expressed as Log 2 (fold change), normalised to housekeeping genes and relative to a normal control group (n=4) which is indicated by the dotted vertical line. Upregulated genes are shown as red, downregulated genes are shown as blue. The significance threshold was $p<0.05$.

3-5: Correlation of inflammasome component mRNA in active UC and active CD														
Inflammasome correlations in active UC														
	IL-1β		*IL-18*		*NLRP1*		*NLRP3*		*NLRP6*		*AIM2*		*ASC*	
	Rs	p	Rs	p	Rs	p	Rs	p	Rs	p	Rs	p	Rs	p
IL-18	0.56	<0.01												
NLRP1	0.48	<0.01	0.17	0.37										
NLRP3	0.78	<0.01	0.66	<0.01	0.57	<0.01								
NLRP6	0.32	0.09	0.31	0.10	0.48	<0.01	0.46	0.01						
AIM2	0.47	<0.01	0.47	<0.01	0.69	<0.01	0.65	<0.01	0.42	0.02				
ASC	0.55	<0.01	0.62	<0.01	0.44	0.02	0.62	<0.01	0.16	0.40	0.72	<0.01		
CASP1	0.63	<0.01	0.79	<0.01	0.19	0.33	0.63	<0.01	0.22	0.25	0.46	0.01	0.57	<0.01
Inflammasome correlations in active CD														
	IL-1β		*IL-18*		*NLRP1*		*NLRP3*		*NLRP6*		*AIM2*		*ASC*	
	Rs	p	Rs	p	Rs	p	Rs	p	Rs	p	Rs	p	Rs	p
IL-18	0.49	0.05												
NLRP1	0.65	<0.01	0.28	0.28										
NLRP3	0.96	<0.01	0.58	0.02	0.74	<0.01								
NLRP6	0.12	0.65	-0.19	0.46	0.07	0.8	0.00	1.0						
AIM2	0.75	<0.01	0.40	0.12	0.69	<0.01	0.83	<0.01	0.16	0.55				
ASC	0.50	0.04	0.34	0.18	0.51	0.04	0.51	0.04	0.45	0.07	0.74	<0.01		
CASP1	0.49	0.05	0.73	<0.01	0.42	0.99	0.57	0.02	0.27	0.30	0.66	<0.01	0.67	<0.01

Rs = 2-tailed Spearman's Rank Correlation Coefficient,
p = probability value associated to the coefficient where $p<0.05$ is considered significant.

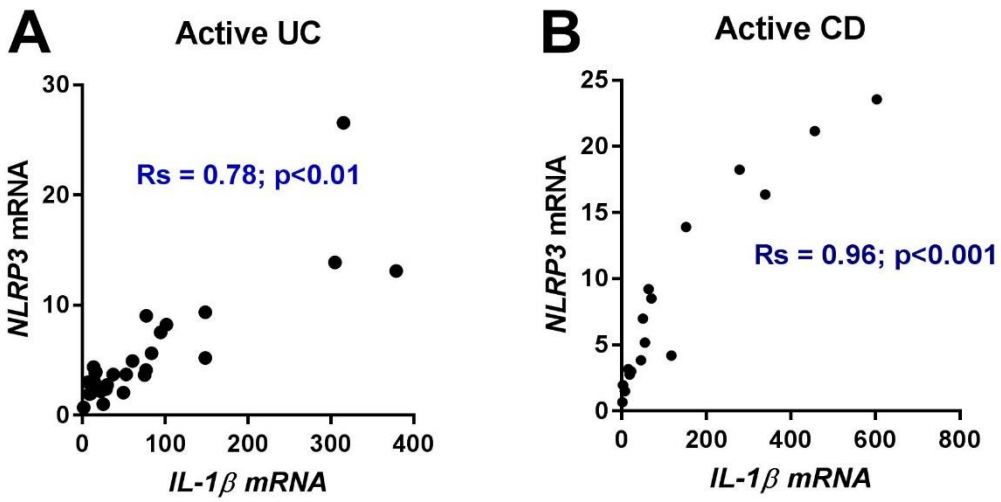


Figure 3-4: Correlation of *NLRP3* to *IL-1β* in active UC and active CD

A) Correlation of *NLRP3* and *IL-1β* relative gene expression in active UC **B)** Correlation of *NLRP3* and *IL-1β* relative gene expression in active CD. Spearman's rank correlation coefficient (Rs) was used to assess the level of association and $p<0.05$ was considered significant.

3.3 DISCUSSION

This study demonstrated the mRNA upregulation of inflammasome forming receptors, inflammasome components and bacterial sensors in active disease which suggests activation of the inflammasome complex. Furthermore, it provided evidence for the upregulation of *NLRP6* (131-fold) in active ileal CD which was not observed in active colonic CD or active UC.

The inflammasome complex is the key regulator of IL-1β production. The demonstrated increase in *IL-1β* gene expression in both active UC and CD is well established and consistent with previous research in human derived material [213-217]. The concomitant expression of *IL-1β* and the inflammasome receptor proteins, *AIM2, NLRP1, NLRP3* and *NLRP6* prevents comparison of the individual inflammasome to the overall production of *IL-1β*. *NLRP3* did however correlate the strongest with *IL-1β* in active UC and CD.

The high expression of *ASC* in ileal CD is likely to be a reflection of the high *NLRP6* and *AIM2* inflammasome activation in this disease group. Previous work in human cell lines have indicated upregulation of *ASC* with inflammasome activation [218, 219]. The increased *CASP1* expression in active UC and CD is well established and consistent with previous studies in human derived material [214, 220].

NLRP6 in ileal CD was shown to increase 131-fold with disease activity. In a paediatric IBD population from Canada the expression of *NLRP6* was found to be low and there was no significant variation between UC and CD [221]. Taken together the NLRP6 discrepancy could be related to age, genetic variability or lifestyle factors and warrants further investigation.

The expression of *IL-18* remain unchanged in both UC and CD despite increased disease activity. Previously, increased serum levels of IL-18 [222] and *IL-18* transcript levels (by semi-quantitative PCR) [206, 223, 224] have been associated with CD.

In the murine system, discrepancies exist regarding the association of NLRP6 to the production of IL-18. Firstly, Nlrp6 deficiency has been associated with low levels of IL-18 [129, 186] and the induction of intestinal IL-18 has been shown to be NLRP6 dependent [225]. In disagreement Normand et al [226] reported no NLRP6-dependent changes in the transcript abundance of IL-18 in tumoral and non-tumoral biopsies procured from Nlrp6+/+ and Nlrp6-/- mice. In agreement with Normand et al [226], this study reports no change in the expression of *IL-18* despite fluctuating *NLRP6* expression, suggesting *IL-18* expression is not *NLRP6* dependent.

The expression of PPAR-γ was found to be reduced in both active UC and active CD, notably in ileal CD. Previously, the expression of *PPAR-γ* has been reported as reduced in active UC and comparable to normal colon in active CD [227, 228]. The notable reduction in active ileal CD suggests possible negative transcriptional control of *NLRP6* by *PPAR-γ* and warrants further investigation.

The demonstrated increase of *NOD2* in active UC is well established and consistent with previous research in cell lines and human derived material [229-232]. Likewise, constitutive expression of *NOD1* has been demonstrated in cell lines and is consistent with the uniform expression observed in CD for the current study [233, 234]. The difference in expression levels of *NOD1* and *NOD2* in UC and CD are in part a reflection of their function. NOD2 is a general bacterial sensor and activates upon exposure to most Gram-negative and Gram-positive bacteria

while NOD1 only senses Gram-negative bacteria but can act as a backup sensor for Gram-negative bacteria that have evaded the TLR system [213, 234]. NOD1 and NOD2 have also been implicated in bacterial clearance pathways downstream of inflammasome activation, such as ROS, reactive nitrogen species (RNS) and autophagy pathways [235]. NOD2 increases the production of antimicrobial α-defensins from paneth cells, and both NOD1 and NOD2 enhance autophagy by co-localising with ATG16LI at the plasma membrane to assist in autophagosome formation [236-238]. Therefore, considering the ways in which NOD1 and NOD2 enhance bacterial killing it is possible that the upregulation of *NOD1* and *NOD2* in UC and upregulation of *NOD2* in active CD is an innate immune response to an increased bacterial burden. The absence of upregulation of *NOD1* in active CD is a reflection of the gut microbiota composition.

In conclusion, results of this study suggest activation of multiple inflammasome complexes in active UC and CD. The identification of NLRP6 as a disease specific marker for ileal CD will be the focus of work presented in Chapter 6.

CHAPTER 4 COLONIC LOCALISATION AND INTRAEPITHELIAL LYMPHOCYTE EXPRESSION OF AIM2

4.1 INTRODUCTION

Microbial DNA is highly immunostimulatory and can trigger multiple pattern recognition receptors and initiate responses via several innate immune pathways [239, 240]. The presence of unmethylated CpG DNA (found in prokaryotic DNA but suppressed in mammalian DNA [241]) in endo-lysosomal compartments activates the membrane bound TLR9 receptor, initiating the downstream activation of inflammatory cytokines such as TNF-α, IL-1, IL-6 and IL-18 via NF-$_k$B transcription and MAPK pathways [51, 242].

Prokaryotic, viral and non-microbial DNA when internalised or delivered into the cytoplasm of cells triggers TLR independent sensing by cytoplasmic DNA sensors, such as absent in melanoma 2 (AIM2). AIM2 is a member of the pyrin and hematopoitic interferon-inducible nuclear proteins (HIN) (PYHIN) receptor family for which there are 4 human members and 11 confirmed mouse members [79]. The structure of AIM2 consists of an N-terminal pyrin domain (PYD) domain attached to a hemopoietic interferon-inducible nuclear protein (HIN-200-C) domain at the C-terminal [80]. The PYD domain forms homotypic interactions with other PYD-containing proteins while the HIN-C domain directly binds to dsDNA of at least 80 base pairs in length, irrespective of its sequence composition [120-122]. Binding occurs at multiple sites along the dsDNA and is through electrostatic attractions between the positively charged HIN domain residues and the dsDNA sugar phosphate backbone. In the absence of DNA, the HIN-C domain interacts with the PYD domain to provide autoinhibition [123].

The presence of cytoplasmic DNA triggers several AIM2 dependent innate immune pathways that result in the production of inflammatory cytokines, anti-viral type I interferons (IFNα/β) [240] and cell death via pyroptotic caspase-1 [243] and the apoptotic executioner caspase-3 [244]. The AIM2 inflammasome pathway activates caspase-1 in an ASC-dependent manner, which enhances the maturation of inflammatory cytokines, IL-1β and IL-18 [120, 121]. Aberrant signaling of the inflammasome complex is thought to be a contributing factor in the development of gastrointestinal disease.

In mouse models utilising DSS and azoxymethane, AIM2 protects the colonic epithelium against tumorigenesis [245]. Furthermore, mutations in the AIM2 gene have been reported in human colorectal cancer with reduced expression of AIM2 in colorectal cancer being associated with a poor prognosis for the patient [246, 247]. Similarly, prostate cancer cells also exhibit reduced AIM2 expression [248]. Interestingly, the overexpression of AIM2 protein in human cancer cell lines has been shown to inhibit cell proliferation and cancer progression [249].

AIM2 is yet to be linked to any inherited autoinflammatory diseases and no gain of function mutations have been identified within the *AIM2* gene [250]. AIM2 does however fall within the susceptibility loci for systemic lupus erythematous and research to determine the contribution of AIM2 to disease development is ongoing [251-253].

It was hypothesised that AIM2 provides cytoplasmic surveillance against foreign DNA and contributes to innate immune defences in the gut by directing downstream inflammatory pathways via the inflammasome complex. In Chapter 3, gene expression data demonstrated the upregulation of *AIM2* in active disease.

Following on from these results the aim of Chapter 4 was to examine the cellular localisation of AIM2 in quiescent and active disease.

4.2 RESULTS

4.2.1 AIM2 ANTIBODY OPTIMISATION

In order to investigate the localisation of AIM2 in colon tissue it was first necessary to establish the efficiency of the AIM2 antibody (ab93015, Abcam, Cambridge, MA, USA) in human control tissue. Using normal human tonsil tissue positive nuclear staining was observed in the germinal centre and non-germinal centre cells with scattered AIM2 expression evident in the stratified squamous epithelium (Figures 4-1 and 4-2). This is consistent with the Abcam product information sheet (ab93015, Abcam, Cambridge, MA, USA) (Appendix 6) which demonstrated positive nuclear staining in formalin fixed paraffin embedded human foetal tonsil using immunohistochemistry.

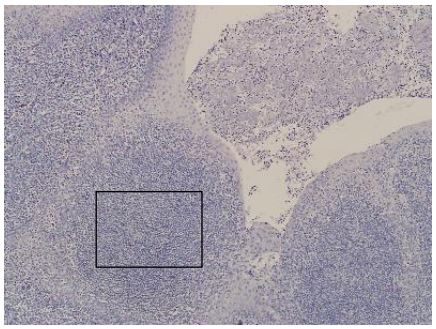
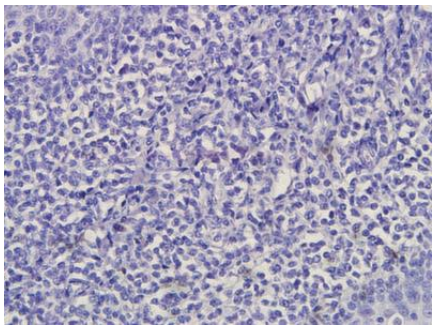
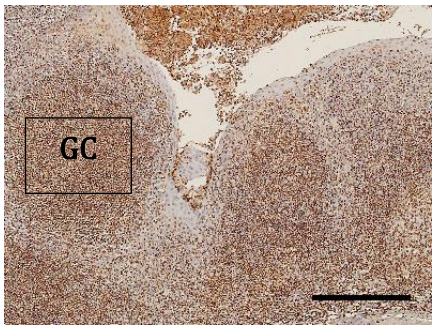
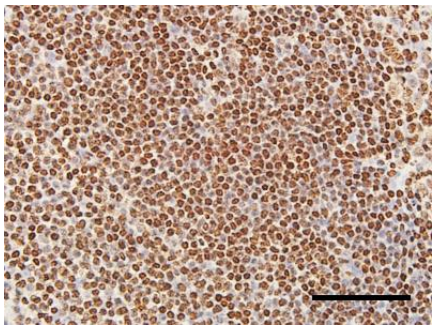
AIM2	100X	400X
Human tonsil Negative control		
Human tonsil Positive control		

Figure 4-1: (previous page) Localisation of AIM2 in normal human tonsil tissue as analysed by immunohistochemistry.

Tissue was paraffin embedded, cut into 5 µm sections and incubated with the AIM2 antibody (ab93015, Abcam, Cambridge, MA, USA) at a dilution of 1:500. Scale bars represent 200 µm for 100X and 50 µm for 400X magnification.

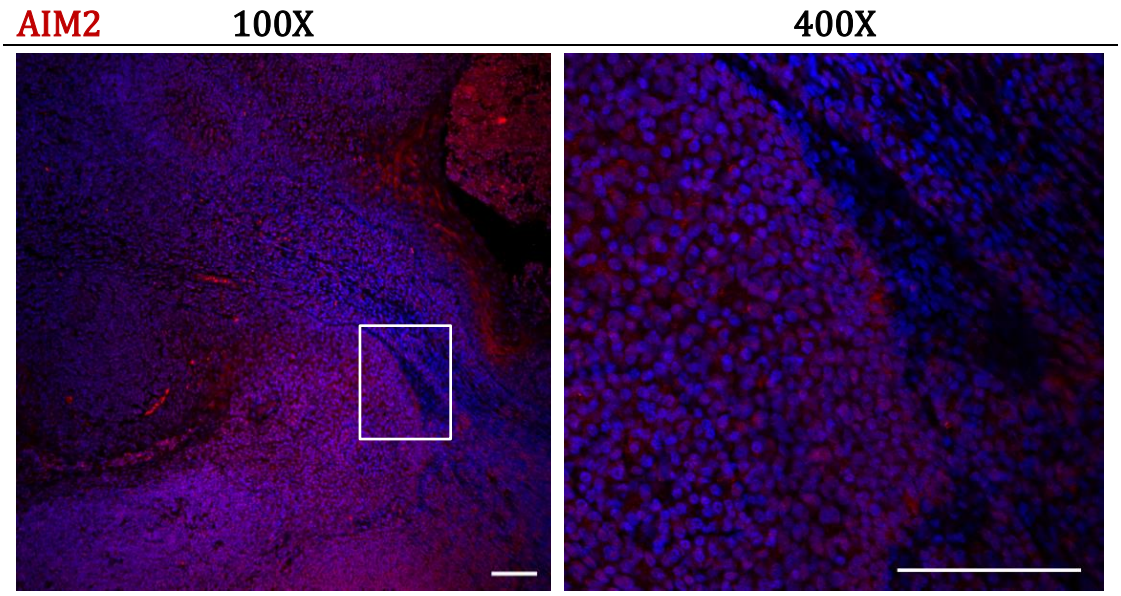


Figure 4-2: Representative immunofluorescence confocal images of AIM2 localisation in normal human tonsil tissue

All sections were paraffin embedded, cut into 5 µm sections, incubated with the AIM2 antibody (ab93015, Abcam, Cambridge, MA, USA, 1:500 dilution) and visualised using Alexa Fluor®647 conjugated goat anti-rabbit IgG (red). Nuclei were stained with 4' ,6 diamidino-2-phenylindole (DAPI, blue). Scale bars represent 100 µm for both the 100X and 400X magnification.

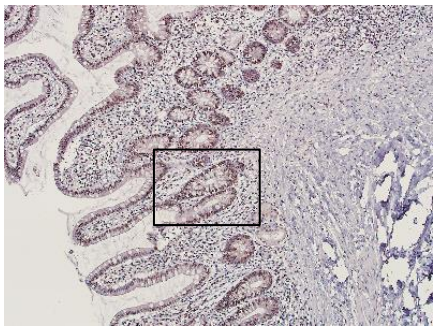
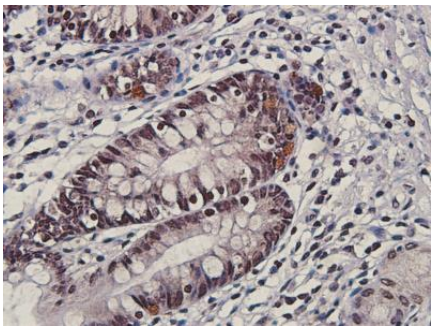
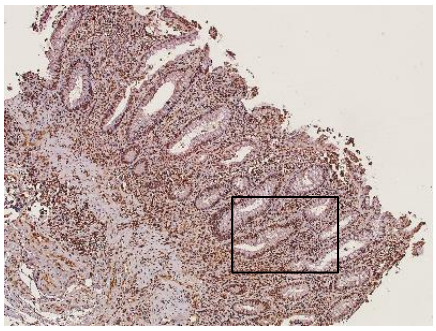
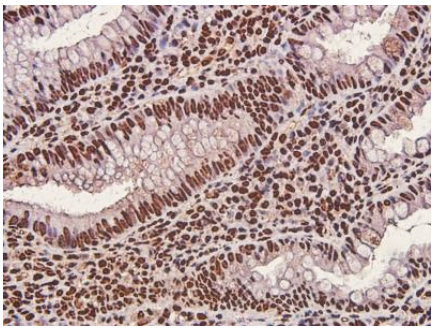
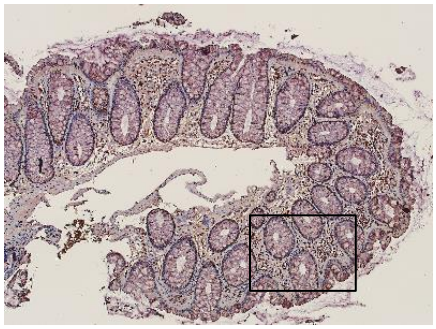
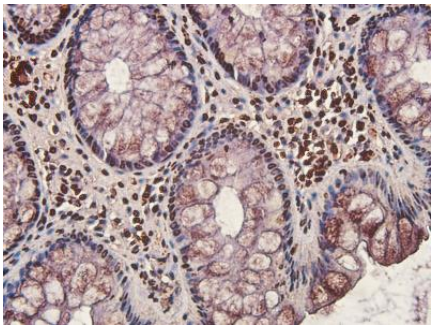
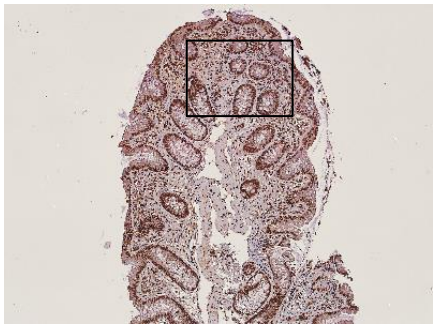
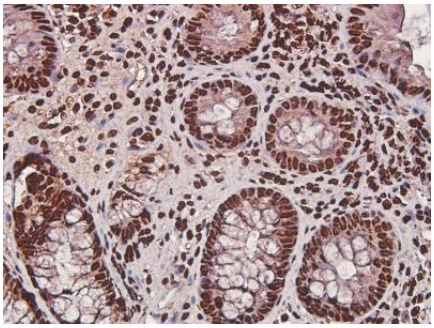
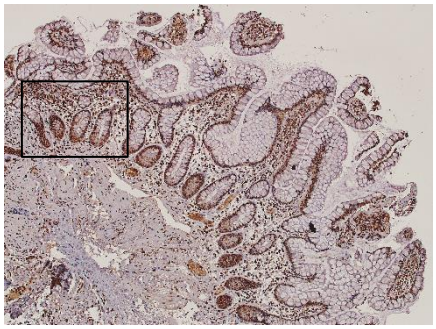
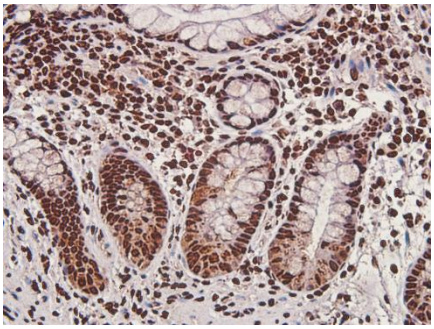
4.2.2 COLONIC LOCALISATION OF AIM2

Immunohistochemistry and immunofluorescence confocal microscopy were used to determine the cellular localisation of AIM2 in active and remission IBD (Figures 4-3 and 4-4). In the normal colon, AIM2 was mainly expressed in the epithelial cell layer at the base of the intestinal crypt with expression disseminated along the crypt length.

In active UC and active CD (ileal and colonic) high AIM2 expression was evident along the entire length of intestinal crypt with intense AIM2 staining present in immune cells of the lamina propria. The expression of AIM2 in UC remission was slightly reduced.

In all IBD and normal colon biopsies prominent AIM2 expression was observed in the intraepithelial lymphocytes lining the intestinal crypts (Figure 4-4, green arrow) which is consistent with work by Vanhove et al [254]. Interestingly, strong AIM2 expression was often observed deep within the crypt and extending into the lumen (Figure 4-4, white arrow).

Quantitatively, AIM2 immunohistochemistry staining was higher in active disease (normal *vs* active UC, $p<0.001$; normal *vs* active ileal CD, $p<0.001$; normal *vs* active colonic CD, $p=0.003$) than in the normal colon and there was a reduction in AIM2 expression with UC remission (UC active *vs* quiescent UC, $p<0.001$). Furthermore, in CD there was no reduction in AIM2 expression with disease remission (Figure 4-5).

AIM2	100X	400X
Normal colon		
Active UC		
Remission UC		
Active ileal CD		
Active ileal CD (ileum biopsy)		

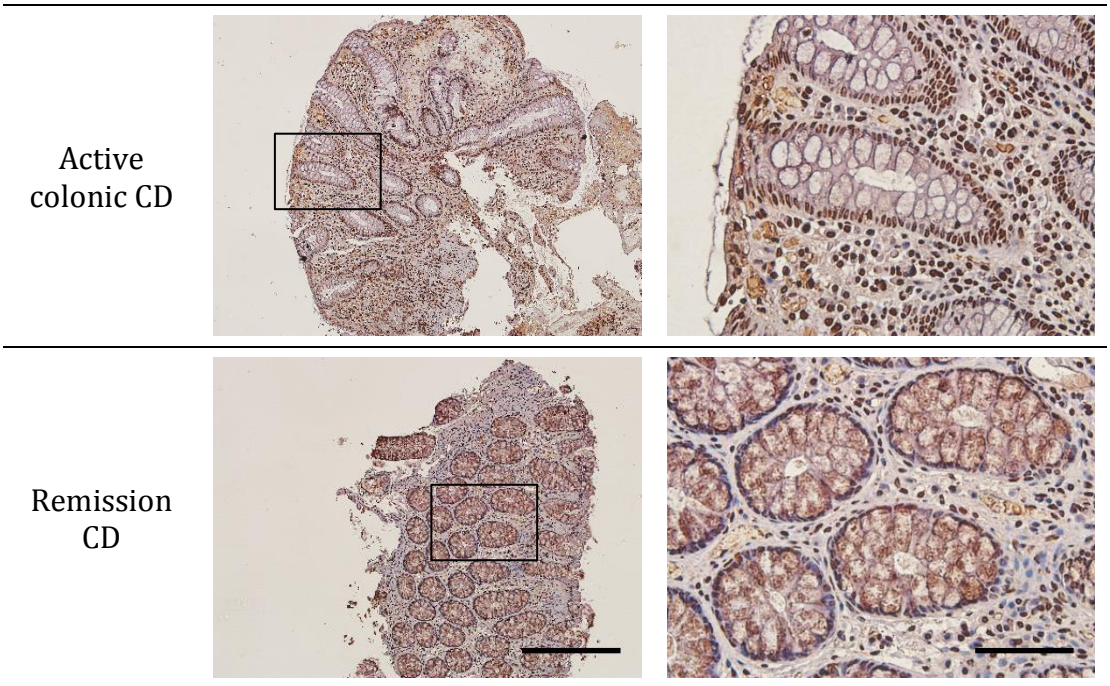
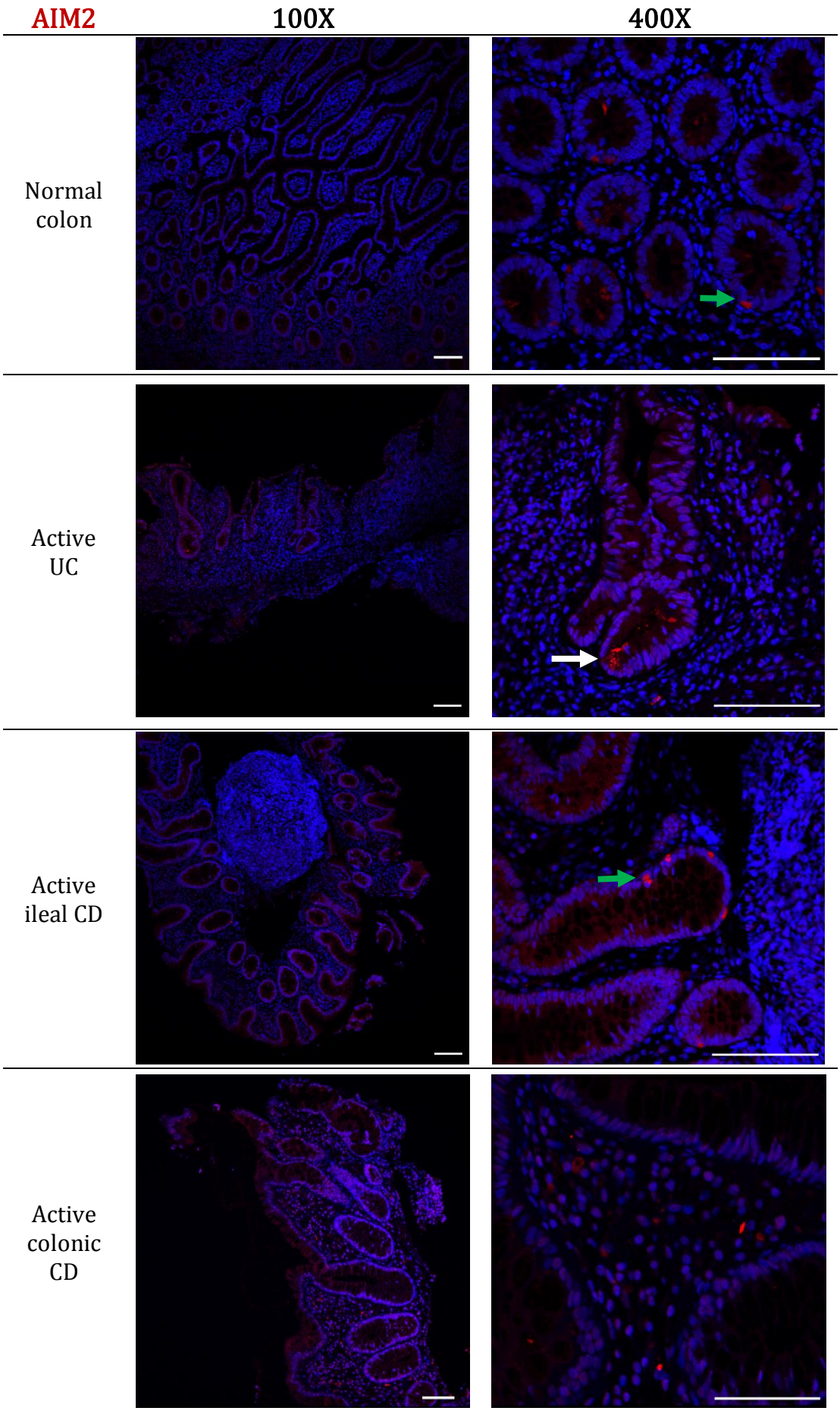


Figure 4-3: Representative immunohistochemistry images of AIM2 expression in normal colon, active UC, remission UC, active ileal and colonic CD and remission CD.

All biopsies were taken from the left colon (unless stated as ileum biopsy), paraffin embedded, cut into 5 µm sections and incubated with AIM2 (ab93015, Abcam Cambridge, MA, USA) at a dilution of 1:500. Scale bars represent 200 µm for 100X and 50 µm for 400X magnification.

Figure 4-4 (next page): Representative immunofluorescence confocal images of AIM2 localisation in normal colon, active UC, active ileal and colonic CD.

All biopsies were taken from the left colon, paraffin embedded, cut into 5 µm sections, incubated with AIM2 (ab93015, Abcam, Cambridge, MA, USA, 1:100 dilution) and visualised using Alexa Fluor®647 conjugated goat anti-rabbit IgG (red). Nuclei were stained with 4' ,6 diamidino-2-phenylindole (DAPI, blue). Green arrows indicate AIM2 positive intraepithelial lymphocyte, white arrow indicates extension of AIM2 expression into crypt lumen. Scale bars represent 100 µm for both the 100X and 400X magnification.


AIM2
100X
400X
Normal colon
Active UC
Active ileal CD
Active colonic CD

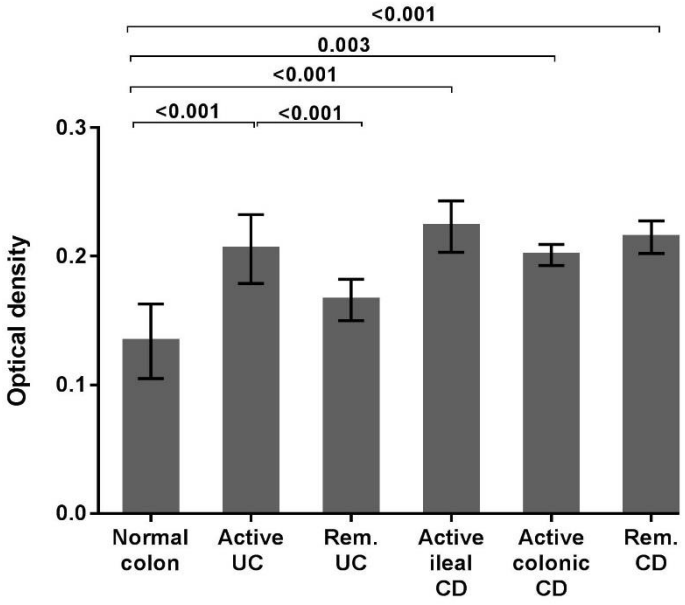


Figure 4-5: Quantification of AIM2 immunohistochemistry staining in biopsy sections from normal and IBD patients

Quantitative analysis of AIM2 expression in sections from normal colon (total number of images analysed=12), active UC (n=104), remission UC (Rem. UC, n=30), active ileal CD (n=24), active colonic CD (n=16) and remission CD (Rem. CD, n=40). Paraffin embedded left colon mucosal biopsies were analysed by immunohistochemistry and the optical intensity of DAB staining due to AIM2 was determined using FIJI software. All data are presented as mean $\pm$ standard deviation. Statistical significance was evaluated using Dunn's multiple comparison one-way analysis of variance (ANOVA). The significance threshold was $p<0.05$.

4.3 DISCUSSION

This study describes the localisation of AIM2 expression in quiescent and active IBD. Abundant AIM2 expression was found in the epithelial cell layer and lamina propria immune cells of colonic and ileum biopsies from active UC and CD. In contrast, the expression of AIM2 in normal colon was localised to the crypt base and disseminated along the length of the colon.

The epithelia cell layer is often the first point of contact for the pathogens that cause disease. Intraepithelial lymphocytes are geared to provide immediate and heightened immune protection to avoid initial entry and spreading of pathogens [255]. The prominent and consistent expression of AIM2 in the intraepithelial lymphocyte cells and protrusion into the lumen suggests AIM2 is a key participant in innate defences during active disease. AIM2 plays an important role in the recognition of viruses and bacteria by the detection of cytosolic dsDNA. *Aim2* deficient mice have demonstrated a critical role for AIM2 in the detection of *Fransicella tularenis,* vaccinia virus, murine cytomegalovirus*, Listeria monocytogenes, Streptococcus pneumonia,* species of *Mycobacterium, Legionella pneumophilia* and *Staphylococcus aureus* [120, 125-127, 256].

AIM2 expression can be rapidly induced by using treatments that promote type 1 interferon (IFN) signaling [250], and previous work has detected basal AIM2 expression in the small intestine, sigmoid colon, rectum, spleen, tonsil, peripheral white blood cells, keratinocytes and testis [80, 249, 257, 258].

The diversity of the gut microbiome tends to be individual-specific and is influenced by lifestyle factors like diet, medication, age and illness [259]. In the normal human gut a dynamic and complex symbiosis exist between the potentially pathogenic bacteria and the “friendly” bugs such as the dominant

Gram-positive *Lactobacilli* and *Bifidobacteria* [260]. *Lactobacilli* and *Bifidobacteria* compete with pathogens like *Bacteriodetes*, *Clostridium, Staphylococcus,* and *Enterobacter* for cell adhesion, hence preventing contact with the intestinal epithelium [261, 262].

In active UC, increased bacterial numbers and alterations in microbial populations are a common finding, and disease remission induces populations comparable to normal gut populations. In contrast, the microbial populations in CD are not altered by disease remission [191]. Interestingly, quantitatively analysis of AIM2 expression in active CD and remission CD were comparable, suggesting the expression of AIM2 is dependent on the local microbial population.

CHAPTER 5 COLONIC LOCALISATION AND SPATIAL INTENSITY RELATIONSHIP OF NLRP3 AND INTERLEUKIN (IL)-1BETA

5.1 INTRODUCTION

An effective host defence and subsequent modulation of the adaptive immune response relies on the induction, production and release of proinflammatory cytokines. One member of the IL-1 family of cytokines, IL-1β is highly inflammatory [263] and classified as a multifunctional cytokine. IL-1β participates in local and systemic responses to injury, infection and inflammation. Clinically, IL-1β has the ability to evoke fever and hypotension [203], and control certain central nervous system functions such as sleep, pain and appetite [70]. Locally, IL-1β can induce cytokine production, enhance T cell activation and antigen recognition, and direct neutrophils to the site of injury or infection [50, 204, 205].

IL-1β is produced as an inactive 31kDa proIL-1β protein in response to TLR activation and enzymatic cleavage is required to generate a bioactive 17kDa IL-1β fragment [132]. The synthesis of a precursor cytokine requiring activation by proteases prevents aberrant secretion of IL-1β.

Several inflammasomes such as, NLRP1, NLRP3, NLRC4, NLRP6, NLRP7, NLRP12, AIM2 and IFI16 have been described for the canonical activation of caspase-1 and maturation of IL-1β [48]. Inflammasome activation is regarded as a two-step process. Transcription and translation of inflammasome components precedes the ligand activation step, which culminates in the assembly of the inflammasome platform and the maturation of IL-1β [95, 96]. Formation of the NLRP3

inflammasome complex occurs in response to a wide range of microbial, environmental and sterile ligands [48, 49]. While the exact mechanisms of ligand activation are yet to be determined it is generally accepted that assembly of the NLRP3 inflammasome occurs in response to host derived factors altered by these agents. The indirect mechanisms include K^+ efflux [87], phagolysosmal destabilisation and release of cathepsins [89], the release of mitochondrial DNA or the mitochondrial phospholipid cardiolipin [90-92], translocation to the mitochondria [88, 93, 94] or the presence of mROS [88].

In addition to inflammasome-dependent production of IL-1β, several cell specific inflammasome-independent processes exist for the activation of IL-1β. IL-1β is a primary product of blood monocytes, tissue macrophages, neutrophils, dendritic cells and to a lesser extent, B lymphocytes and Natural Killer cells [264]. In blood monocytes, caspase-1 is constitutively expressed and the production of active IL-1β occurs via transcriptional control of proIL-1β. This mechanism is thought to aid host defences by reducing immune response time [60].

The zinc-dependent metalloproteinases, meprin A and meprin α are highly expressed at the brush-border membranes of the kidney and intestine and are capable of generating biologically active IL-1β [265]. During acute inflammation, neutrophil and macrophage derived serine proteases, proteinase 3, elastase and cathepsin G can process proIL-1β to biologically active IL-1β [60-63]. Indeed, microbes such as *Candida albicans* and *Staphylococcus spp.* can themselves produces proteases, which possess the ability to cleave IL-1β [266, 267].

Taken altogether, IL-1β is a highly inflammatory cytokine and numerous mechanisms control the production and activity of IL-1β. In the gut it is hypothesised that the NLRP3 inflammasome directs downstream innate immune

defences by regulating the maturation of the IL-1β cytokine. In Chapter 3, gene expression data demonstrated the upregulation of *NLRP3* and *IL-1β* in active disease. Following on from these results the aim of Chapter 5 was to examine the cellular localisation of NLRP3 and IL-1β, and the spatial interaction of NLRP3 and IL-1β in active disease.

5.2 RESULTS

5.2.1 NLRP3 AND IL-1B ANTIBODY OPTIMISATION

In order to investigate the localisation of NLRP3 and IL-1β in colon tissue it was first necessary to establish the efficiency of the primary antibodies in human control tissues. The NLRP3 antibodies (ab17267 and ab16097, Abcam, Cambridge, MA, USA) demonstrated positive staining in the cytoplasmic regions of the stratified squamous epithelial layer of normal human tonsil (Figures 5-1 and 5-2). In agreement, distinct NLRP3 expression has previously been reported for the stratified non-keratinizing squamous epithelium of the oral and oesophageal mucosa [268].

The IL-1β antibody (ab9722, Abcam, Cambridge, MA, USA) demonstrated positive cytoplasmic staining for the circulating immune cells, such as macrophages, lymphocytes and dendritic cells, localised to the paracortical zone of human lymph node tissue (Figures 5-1 and 5-2). In agreement, positive IL-1β staining has previously been reported within the germinal centre, paracortical and mantle zones of human lymph node tissue during infection [269].

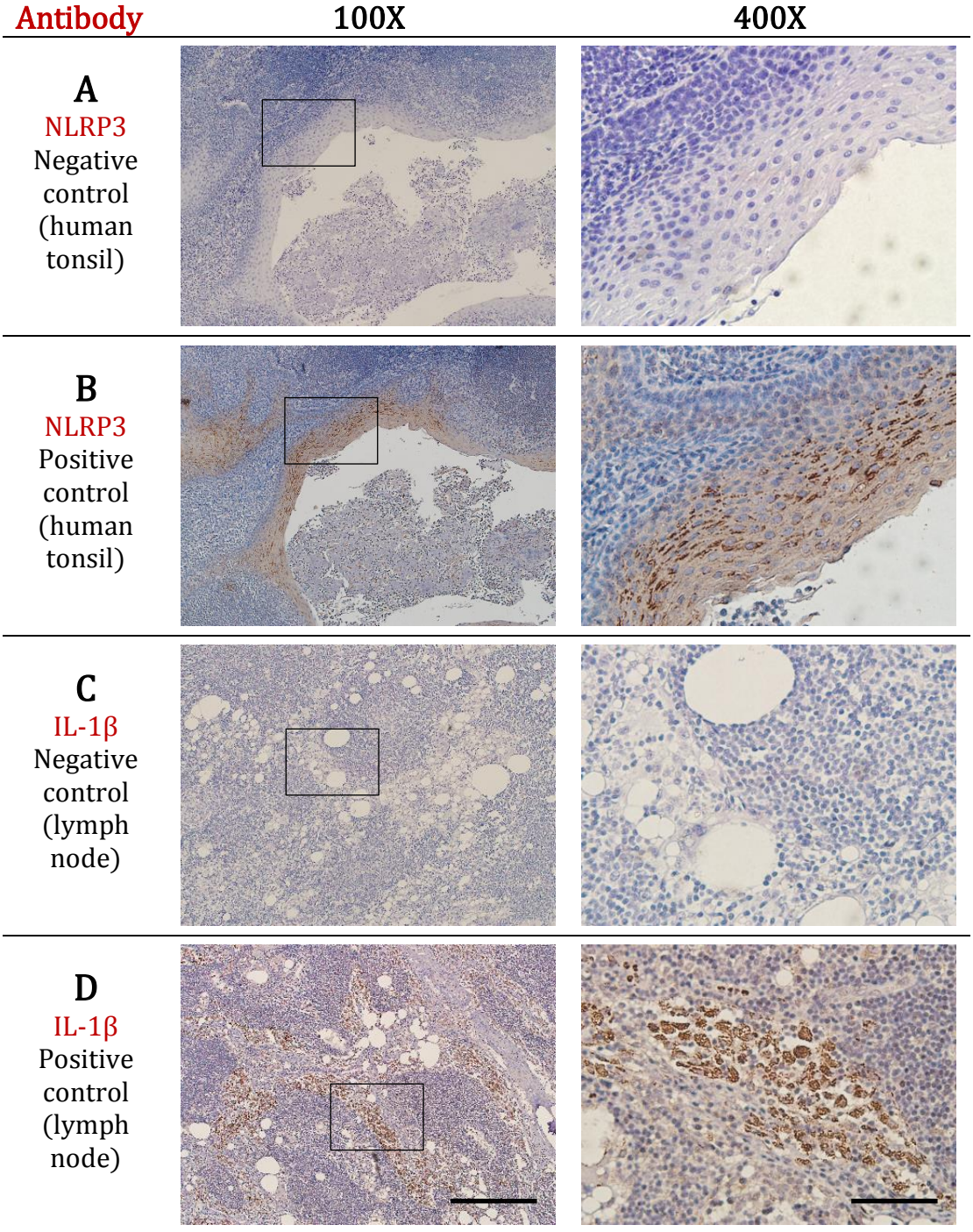


Figure 5-1: Representative immunohistochemistry images of NLRP3 and IL-1β localisation in human control material

All sections were paraffin embedded, cut into 5 µm sections and mounted on superfrost plus slides. **A and B)** Localisation of NLRP3 (ab17267, Abcam, Cambridge, MA, USA) in human tonsil, diluted 1:300. **C and D)** Localisation of IL-1β (ab9722, Abcam, Cambridge, MA, USA) in human lymph node tissue, diluted 1:300. Scale bars represent 50 µm for the 400X and 200 µm for the 100X magnification.

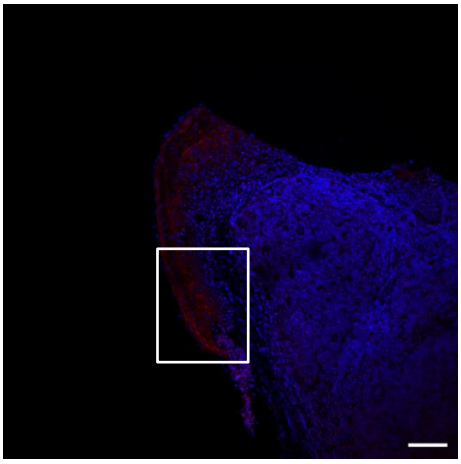
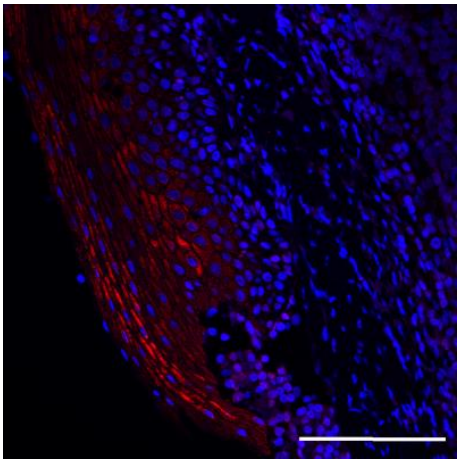
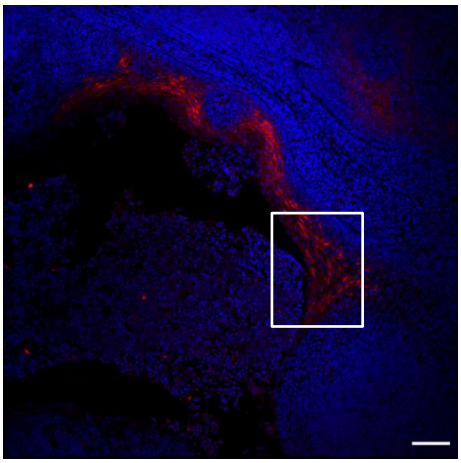
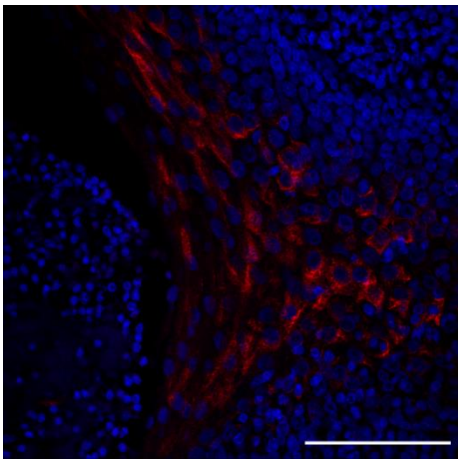
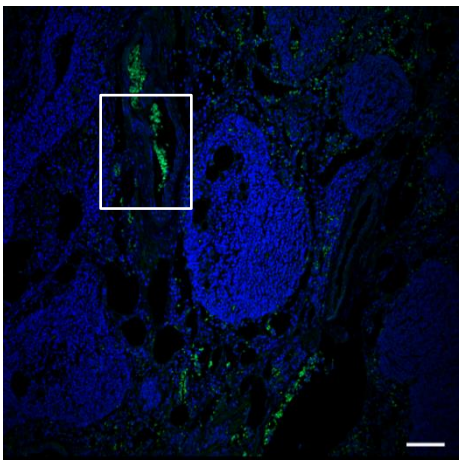
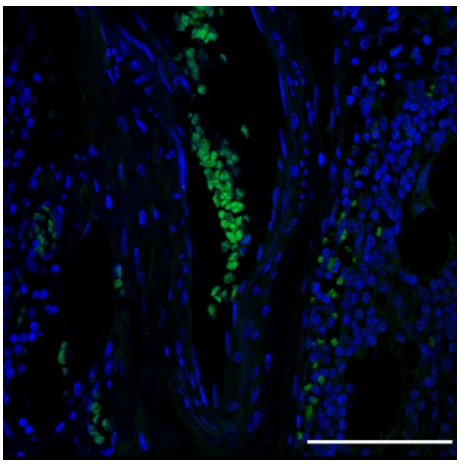
Antibody	100X	400X
A NLRP3 (ab17267) Normal tonsil		
B NLRP3 (ab16097) Normal tonsil		
C IL-1β (ab9722) Lymph node		

Figure 5-2 (previous page): Representative immunofluorescence confocal images of NLRP3 and IL-1β localisation in human control material

All sections were paraffin embedded, cut into 5 μm sections and mounted on superfrost plus slides. **A)** Localisation of NLRP3 (ab17267, Abcam, Cambridge, MA, USA) in normal human tonsil, diluted 1:100 and visualised using Alexa Fluor®647 conjugated goat anti-mouse IgG (red). **B)** Localisation of NLRP3 (ab16097, Abcam, Cambridge, MA, USA) in normal human tonsil, diluted 1:100 and visualised using Alexa Fluor®647 conjugated goat anti-mouse IgG (red). **C)** Localisation of IL-1β (ab9722, Abcam, Cambridge, MA, USA) in human lymph node tissue, diluted 1:100 and visualised using Alexa Fluor®555 conjugated goat anti-rabbit IgG (green). Nuclei were stained with 4' ,6 diamidino-2-phenylindole (DAPI, blue). Scale bars represent 100 μm for both the 100X and 400X magnification.

5.2.2 COLONIC LOCALISATION OF NLRP3

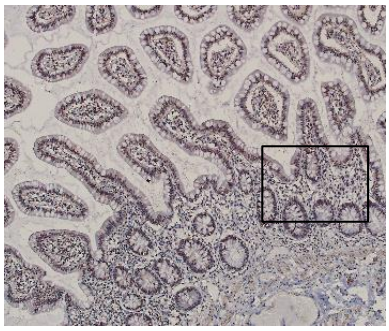
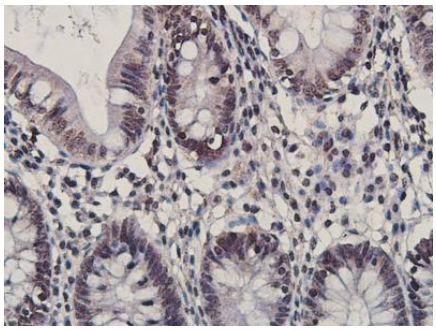
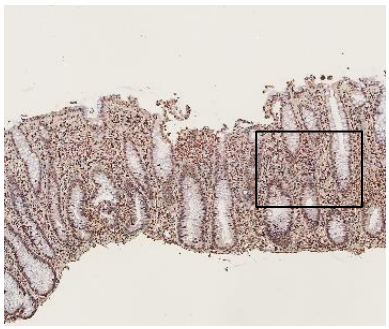
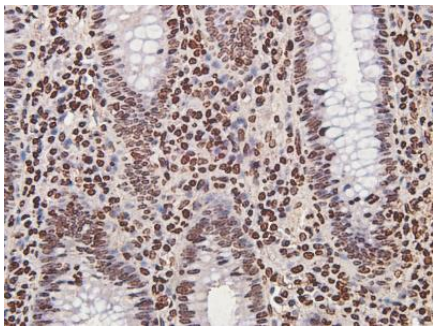
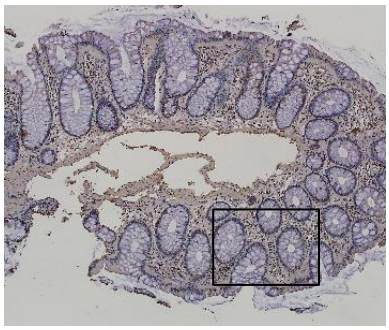
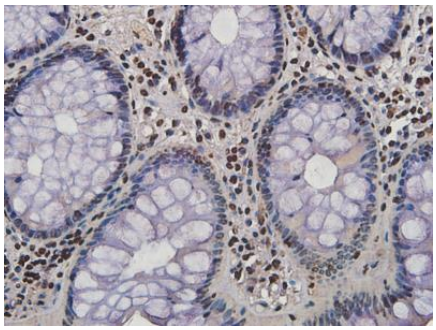
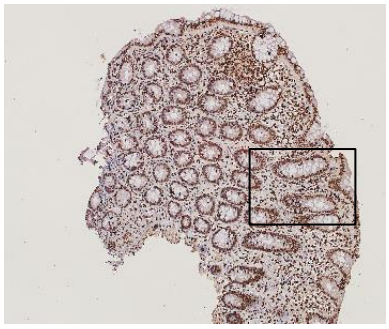
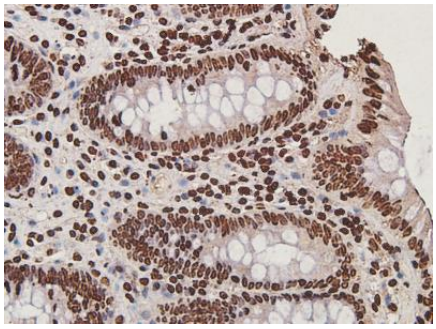
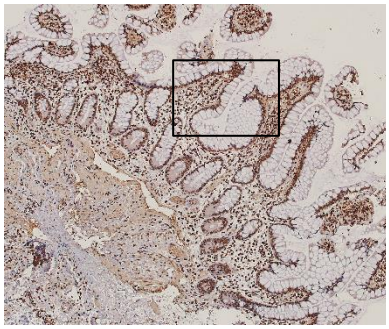
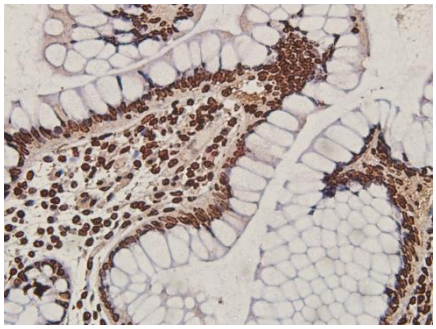
The expression of NLRP3 increased with disease activity in UC and CD (Figures 5-3 and 5-4). In active UC, prominent cytoplasmic staining was present in the neutrophils and other immune cells of the lamina propria while diffuse staining was evident in the epithelial cell region. Consistent with pathology reports, biopsy sections from active UC patients were characterised by an influx of lamina propria immune cells concentrated around regions of crypt distortion or mucosal inflammation.

For active ileal and colonic CD, the reported influx of lamina propria immune cells seen in active UC was not evident, however the intensity of staining within lamina propria immune cells was comparable. Moderate cytoplasmic staining of NLRP3 was also evident in the epithelial cell layer including around goblet cells. Similar expression patterns were observed for both the colon and ileum biopsies in active ileal CD.

In the normal colon, scattered lamina propria immune cells demonstrated cytoplasmic NLRP3 expression and minimal NLRP3 staining was noted in the epithelial cell layer of the intestinal crypts. Remission UC and CD had similar NLRP3 staining patterns with the occasional lamina propria immune cells presenting with NLRP3 expression while epithelial NLRP3 expression was unremarkable.

Quantitative analysis of NLRP3 staining indicated that NLRP3 expression is higher in active UC (normal *vs* active UC, $p<0.001$) and active ileal CD (normal *vs* active ileal CD, $p=0.004$) than in the normal colon (Figure 5-7A). Remission of both UC (active UC *vs* remission UC, $p<0.001$) and ileal CD (active ileal CD *vs* remission CD, $p=0.004$) but not colonic CD result in a reduction of NLRP3

expression. Interestingly, NLRP3 expression levels for disease in remission are similar to normal colon levels which is in contrast to the reduction of lL-1β expression seen when UC or CD experience disease remission (Figure 57-B).

NLRP3	100X	400X
Normal colon		
Active UC		
Remission UC		
Active ileal CD		
Active ileal CD (ileum biopsy)		

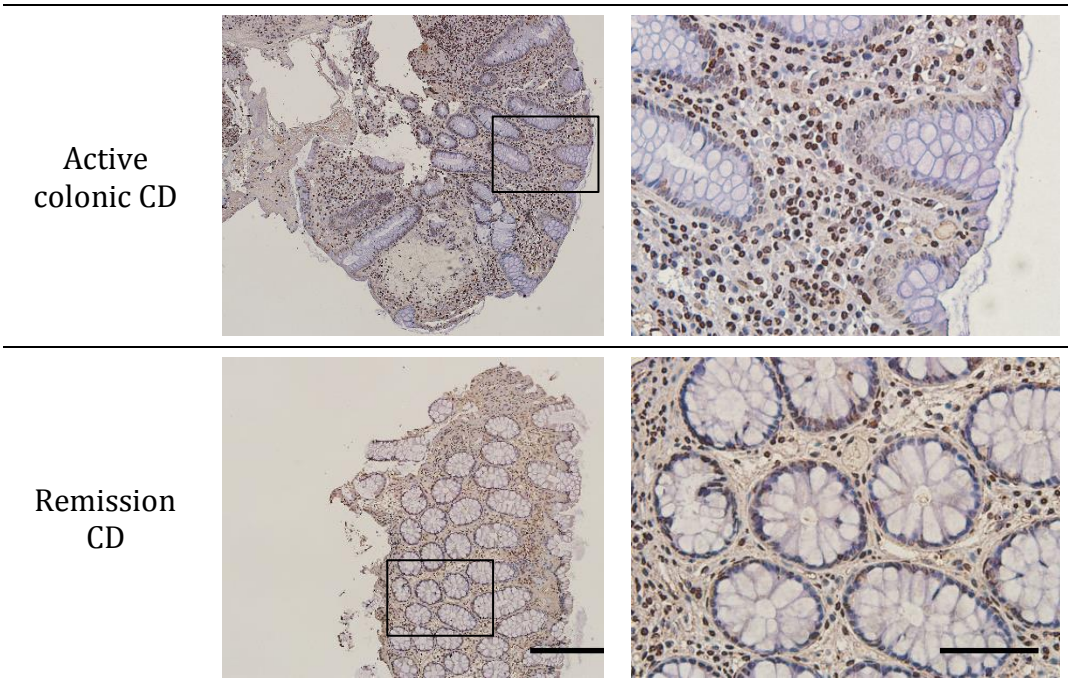
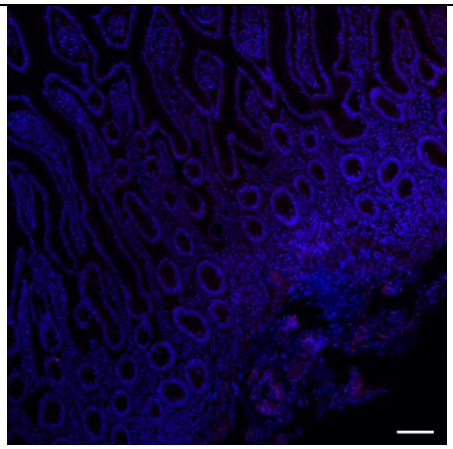
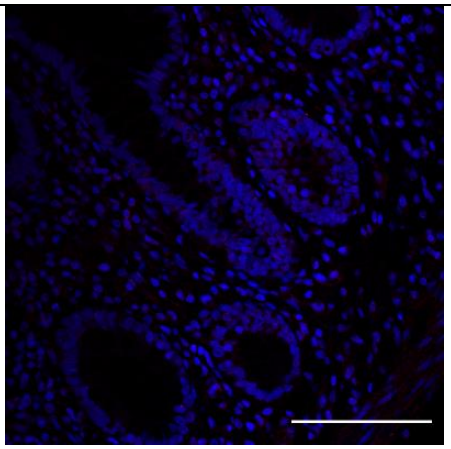
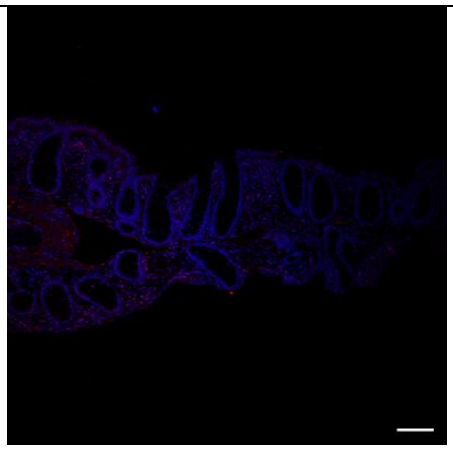
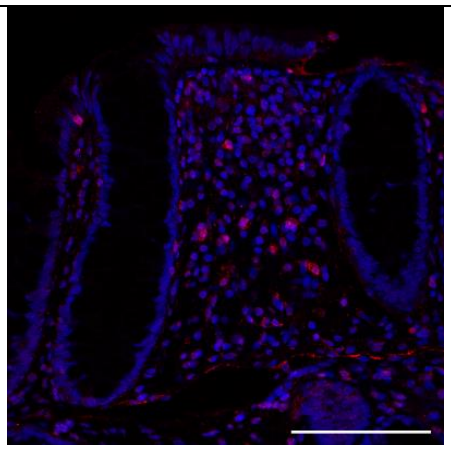
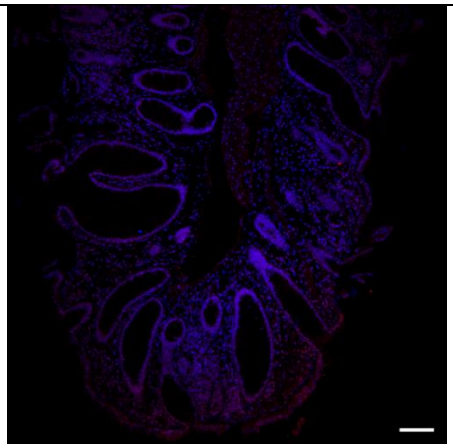
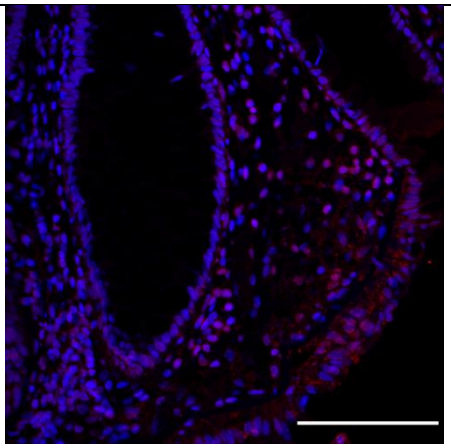
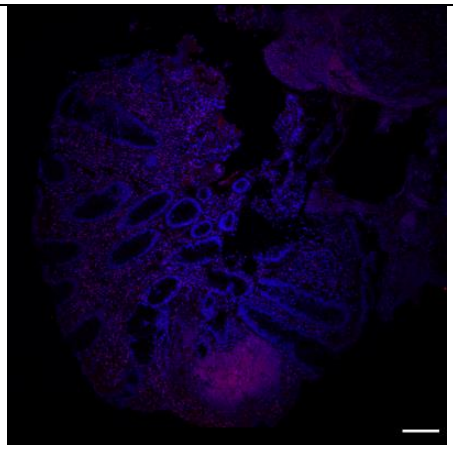
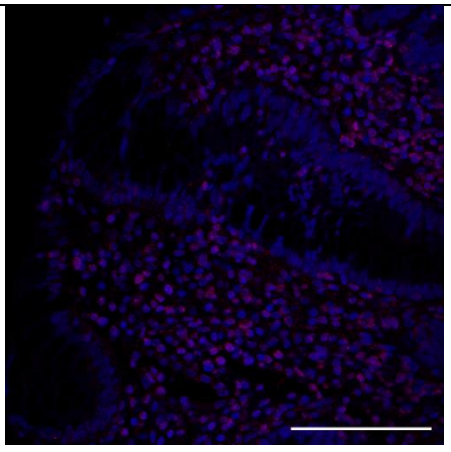


Figure 5-3: Representative immunohistochemistry images of NLRP3 expression in normal colon, active and remission UC, active ileal and colonic CD and remission CD.

All biopsies were taken from the left colon (unless otherwise stated as ileum biopsy), paraffin embedded, cut into 5 µm sections and incubated with NLRP3 (ab17267, Abcam, Cambridge, MA, USA) at a dilution of 1:300. Scale bars represent 200 µm for 100X and 50 µm for 400X magnification.

Figure 5-4 (next page): Representative immunofluorescence confocal images of NLRP3 expression in normal colon, active UC and active ileal and colonic CD.

All biopsies were taken from the left colon, paraffin embedded, cut into 5 µm sections and incubated with NLRP3 (ab17267, Abcam, Cambridge, MA, USA) at a dilution of 1:100 and visualised using Alexa Fluor®647 conjugated goat anti-mouse IgG (red). Nuclei were stained with 4' ,6 diamidino-2-phenylindole (DAPI, blue). Scale bars represent 100 µm for both the 100X and 400X magnification.

NLRP3	100X	400X
Normal colon		
Active UC		
Active ileal CD		
Active colonic CD		

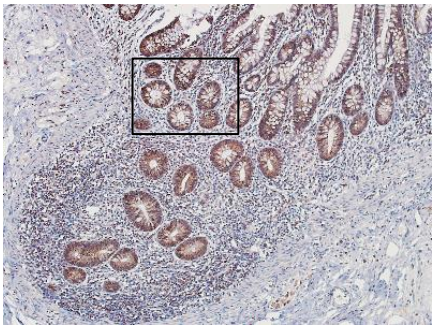
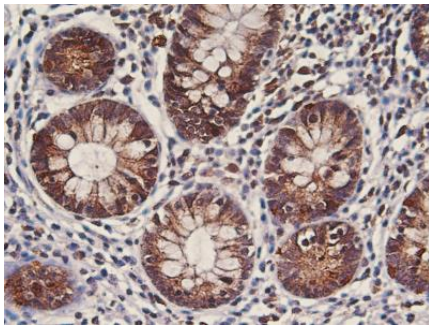
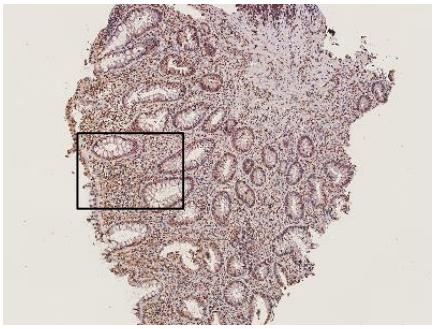
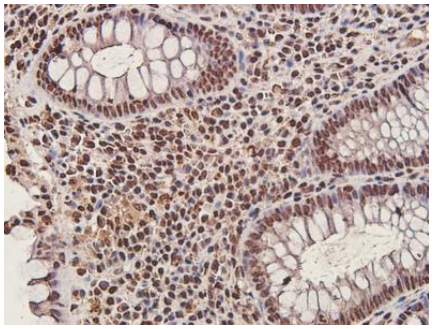
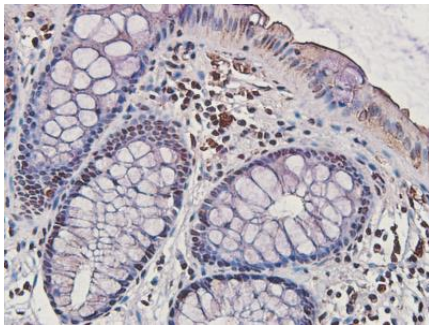
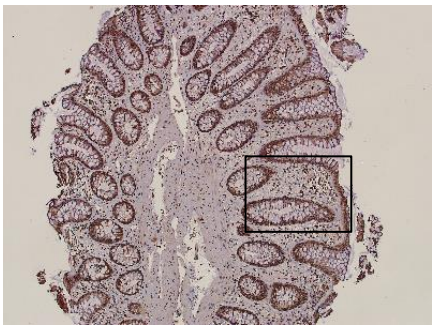
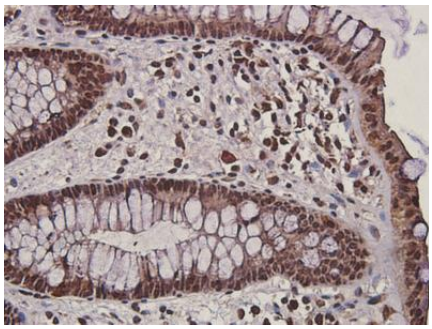
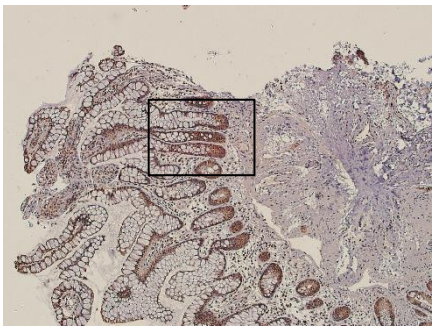
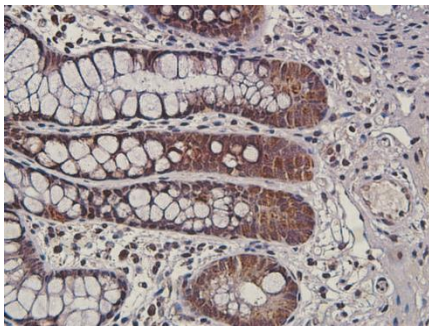
5.2.3 COLONIC LOCALISATION OF IL-1BETA

The expression of IL-1β was highly variable across all IBD phenotypes (Figures 5-5 and 5-6). In the normal colon, high expression of IL-1β was observed within the epithelial cell layer of the mucosal crypts, including the cytoplasm of intestinal goblet cells. Unremarkable was the occasional immune cell within the lamina propria demonstrating cytoplasmic and/or nuclear IL-1β staining.

In active UC, there was a marked increase in the number of lamina propria immune cells, which was consistent with histology pathology reports. Notable was the high proportion of lamina propria cells exhibiting dense cytoplasmic and nuclear IL-1β expression. This high IL-1β expression was not observed for cells within the epithelial layer of the intestinal crypts. Remission UC demonstrated a marked decrease in the intensity of IL-1β staining within cells of the lamina propria with no staining evident in the epithelial cell layer or near goblet cells.

A slight increase in the number of lamina propria immune cells was observed in active ileal and colonic CD and is consistent with pathology reports. Cytoplasmic IL-1β staining was evident in a high proportion of lamina propria immune cells. Remarkable IL-1β expression was observed in the epithelial cells layer, notably the cytoplasm of goblet cells for ileal CD but not colonic CD. For active ileal CD, the expression of IL-1β was similar in both colon and ileum biopsies. In remission CD, IL-1β expression was unremarkable with only scattered staining evident in the lamina propria immune cells. The slightly diminished sensitivity in IL-1β staining observed in sections stained by immunofluorescence was attributed to the inability of immunofluorescence imagery to distinguish between cell borders and background tissue.

Quantitative analysis of IL-1β staining indicated that IL-1β expression is higher in active UC (normal *vs* active UC, p=0.002) and active ileal CD (normal *vs* active ileal CD, p=0.002) but not colonic CD (normal *vs* active colonic CD, p>0.99, not significant). A reduction in the expression of IL-1β was evident when both UC (active UC *vs* remission UC, p<0.001) and ileal CD (active ileal CD *vs* remission CD, p<0.001; active colonic CD *vs* remission CD, p=0.87) were in clinical remission (Figure 5-7B). Interestingly, disease in remission IL-1β levels were lower than those seen in the normal colon (normal colon *vs* UC remission, p=0.004; normal colon *vs* CD remission, p=0.149, not significant).

IL-1β	100X	400X
Normal colon		
Active UC		
Remission UC		
Active ileal CD		
Active ileal CD (ileum biopsy)		

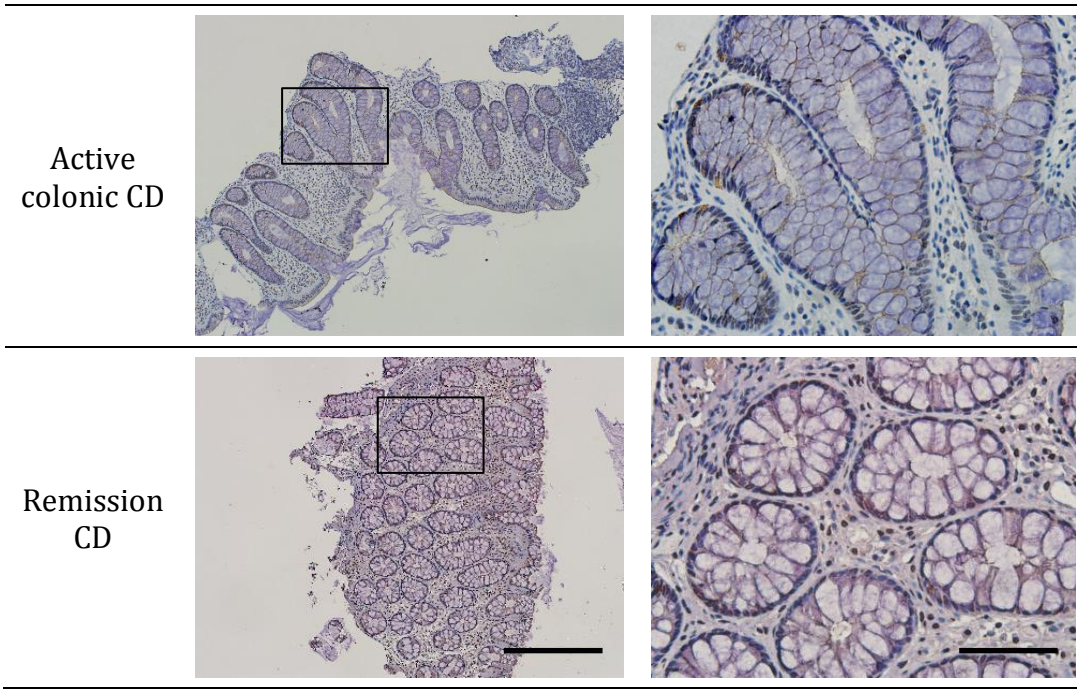
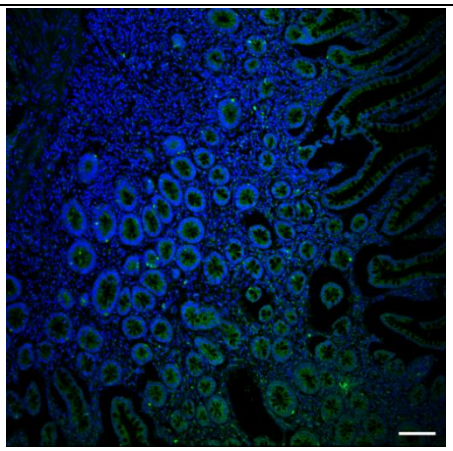
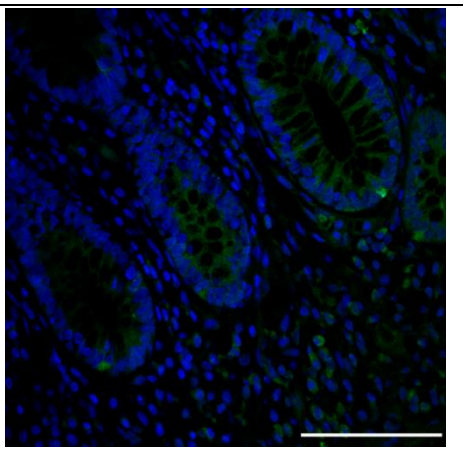
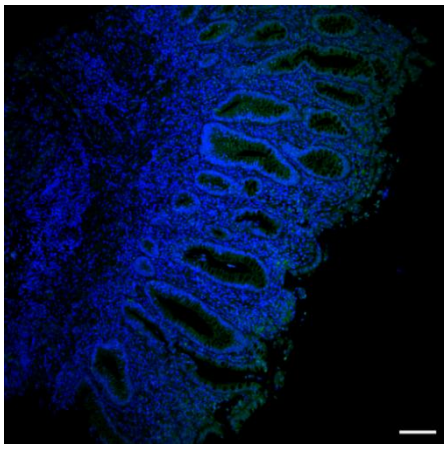
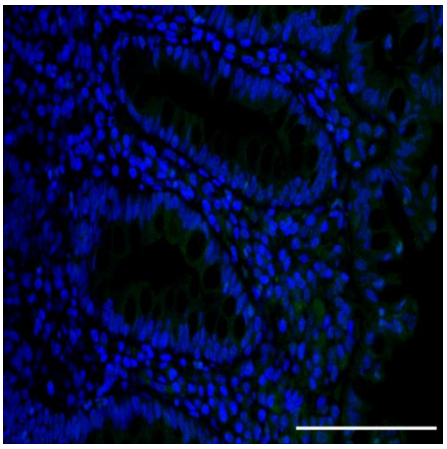
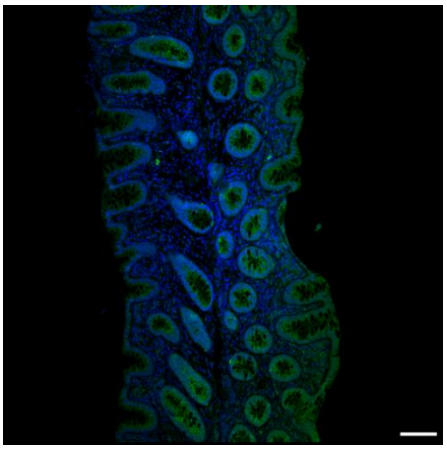
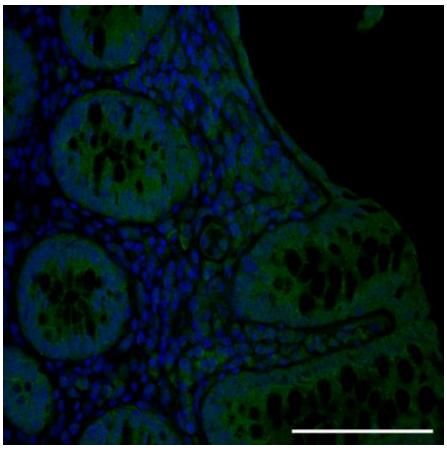
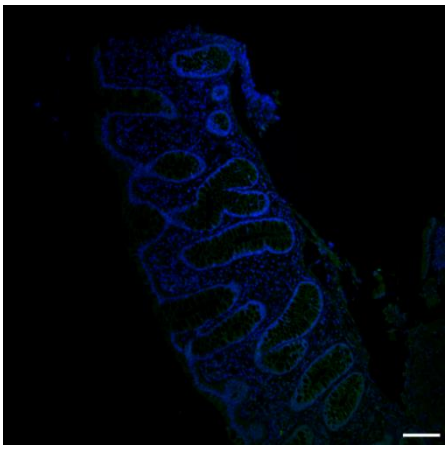
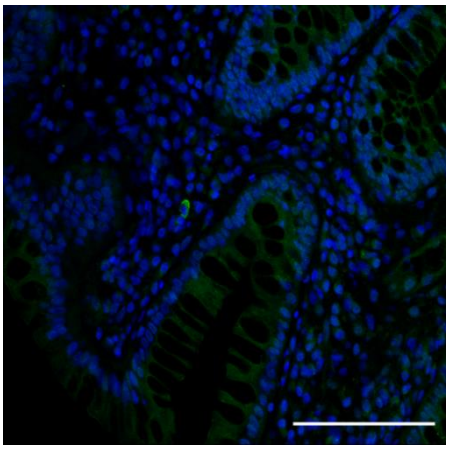


Figure 5-5: Representative immunohistochemistry images of IL-1β expression in normal colon, active UC, remission UC, active ileal and colonic CD and remission CD.

All biopsies were taken from the left colon (unless stated as ileum biopsy), paraffin embedded, cut into 5 μm sections and incubated with IL-1β (ab9722, Abcam, Cambridge, MA, USA) at a dilution of 1:300. Scale bars represent 50 μm for 400X and 200 μm for 100X magnification.

Figure 5-6 (next page): Representative immunofluorescence confocal images of IL-1β expression in normal colon, active UC and active ileal and colonic CD.

All biopsies were taken from the left colon, paraffin embedded, cut into 5 μm sections and incubated with IL-1β (ab9722, Abcam, Cambridge, MA, USA. 1:100 dilution) and visualised using Alexa Fluor®555 conjugated goat anti-rabbit IgG (green). Nuclei were stained with 4' ,6 diamidino-2-phenylindole (DAPI, blue). Scale bars represent 100 μm for both the 100X and 400X magnification.

IL-1β	100X	400X
Normal colon		
Active UC		
Active ileal CD		
Active colonic CD		

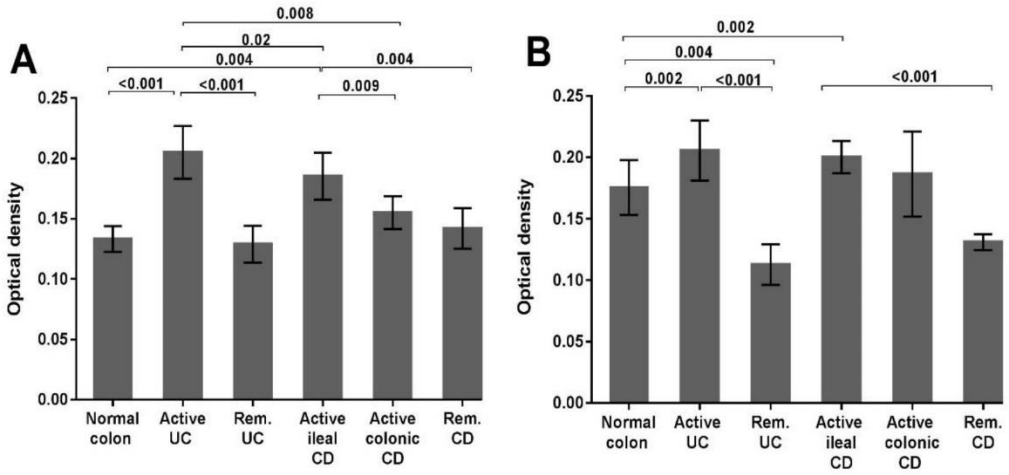


Figure 5-7: Quantification of NLRP3 and IL-1β immunohistochemistry staining in biopsy sections from normal and IBD patients

A) Quantitative analysis of NLRP3 expression in sections from normal colon (total number of images analysed=10), active UC (n=91), remission UC (Rem. UC, n=21), active ileal CD (n=25), active colonic CD (n=17) and remission CD (Rem. CD, n=18). **B)** Quantitative analysis of IL-1β expression in sections from normal colon (n=24), active UC (n=46), remission UC (Rem. UC, n=20), active ileal CD (n=48), active colonic CD (n=16) and remission CD (Rem. CD, n=18). Paraffin embedded left colon mucosal biopsies were analysed by immunohistochemistry and the optical intensity of DAB staining due to primary antibody was determined using FIJI software. All data are presented as mean ± standard deviation. Statistical significance was evaluated using Dunn's multiple comparison one-way analysis of variance (ANOVA). The significance threshold was $p<0.05$.

5.2.4 COLOCALISATION OF NLRP3 AND IL-1BETA IN ACTIVE UC AND CD

Immunofluorescence confocal microscopy was performed to assess the spatial relationship of NLRP3 to IL-1β within the cells of the lamina propria. Manders coefficients (M1 and M2) were used to evaluate the reciprocal association ratio between fluorescence markers with values ranging from 0.5 to 1.0 indicating a positive association. Pearson's correlation coefficient was used to describe the correlation of the intensity distribution between channels. Values range from -1 to 1 and indicate the strength of the negative or positive correlation [270].

For normal colon, a high proportion of colocalisation between NLRP3 and IL-1β was evident within the lamina propria immune cells (Figure 5-8). Quantification of Manders coefficients confirmed a positive association between NLRP3 and IL-1β (M1, NLRP3:IL-1β $= 0.81 \pm 0.06$; M2, IL-1β:NLRP3 $= 0.82 \pm 0.06$) which indicates a similar contribution of one to the other (Figure 5-9A).

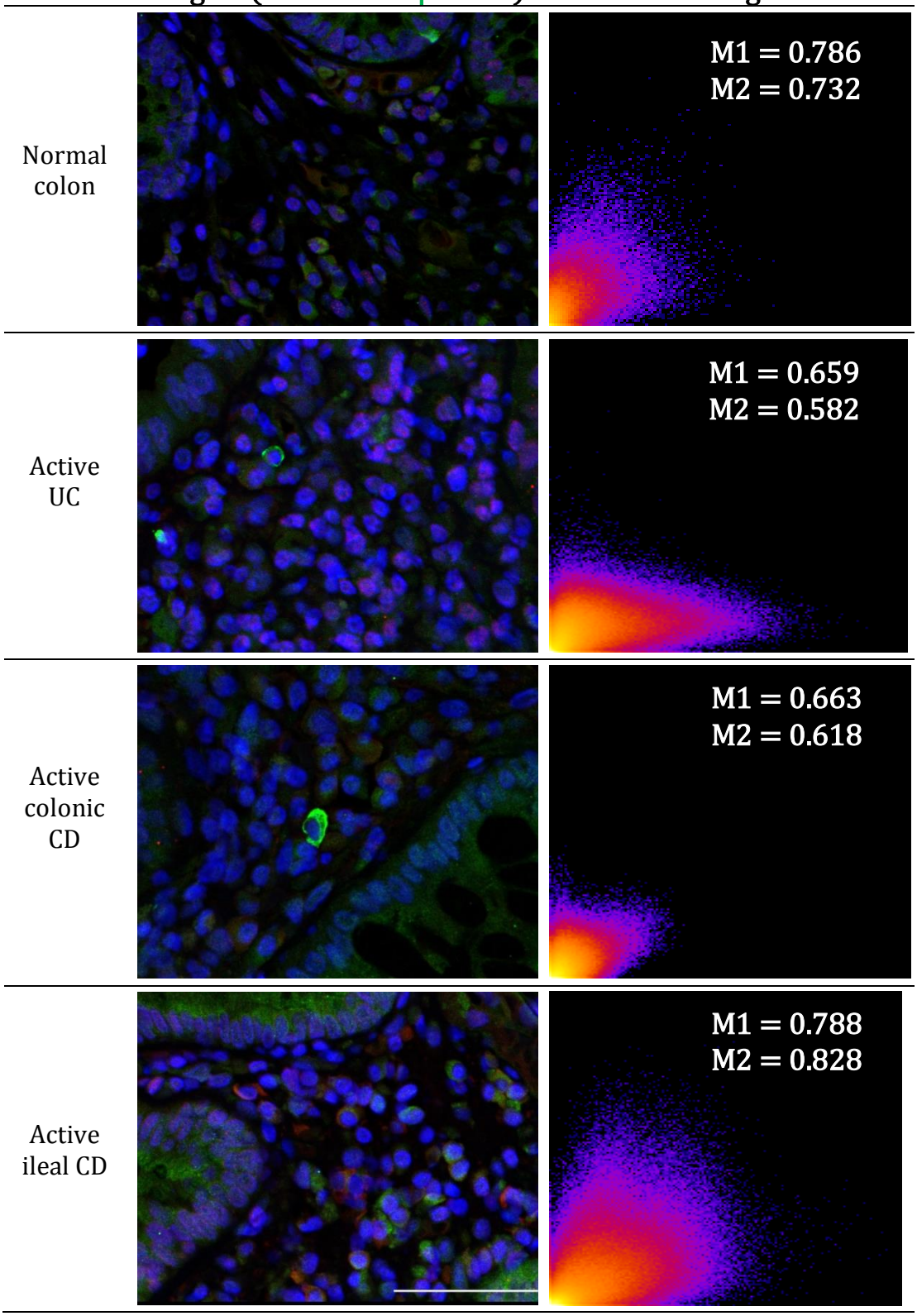
Manders coefficients demonstrated less colocalisation of NLRP3 to IL-1β in active UC (M1, NLRP3:IL-1β $= 0.67 \pm 0.04$; M2, IL-1β:NLRP3 $= 0.61 \pm 0.09$) when compared to the normal colon biopsies. Interestingly the appearance of a y-dominated scattergram suggests increased IL-1β production without concomitant increases in NLRP3 (Figure 5-8). The lack of correlation between NLRP3 and IL-1β in active UC is confirmed by the near zero (0.03) Pearson correlation coefficient (Figure 5-10).

For active CD, the colocalisation of NLRP3 and IL-1β was stronger in ileal CD (M1, NLRP3:IL-1β $= 0.78 \pm 0.05$; M2, IL-1β:NLRP3 $= 0.80 \pm 0.05$; Pearson's correlation coefficient $= 0.29$) than in colonic CD (M1, NLRP3:IL-1β $= 0.63 \pm$

0.05; M2, IL-1β:NLRP3 $= 0.66 \pm 0.05$; Pearson's correlation coefficient $= 0.22$) (Figure 5-9C, Figure 5-9D and Figure 5-10).

Figure 5-8 (next page): Representative co-immunostaining images of NLRP3 (red) and IL-1b (green) in normal colon and active UC and active CD.

Parrafin embedded colon biopsies were simultaneously stained with NLRP3 (ab16097, Abcam, Cambridge, MA, USA) and IL-1β (ab9722, Abcam, Cambridge, MA, USA) and visualised using Alexa Fluor®647 conjugated mouse anti-goat (red) and Alexa Fluor® 555 conjugated rabbit anti-goat (green) respectively. Nuclei were stained with 4' ,6 diamidino-2-phenylindole (DAPI, blue). The 2D histogram visualises the overall relationship of channel intensities for homologous pixels. The coordinates of the scattergram are the channel (CH) intensities of NLRP3 (red-CH1-x) and IL-1β (green-CH2-y) within each pixel. The value at each location indicates the incidence of the combination. Scale bar $= 50$ μm for 400X magnification.


Merged (NLRP3 IL-1β DAPI)
2D histogram
Normal colon
M1 = 0.786
M2 = 0.732
Active UC
M1 = 0.659
M2 = 0.582
Active colonic CD
M1 = 0.663
M2 = 0.618
Active ileal CD
M1 = 0.788
M2 = 0.828

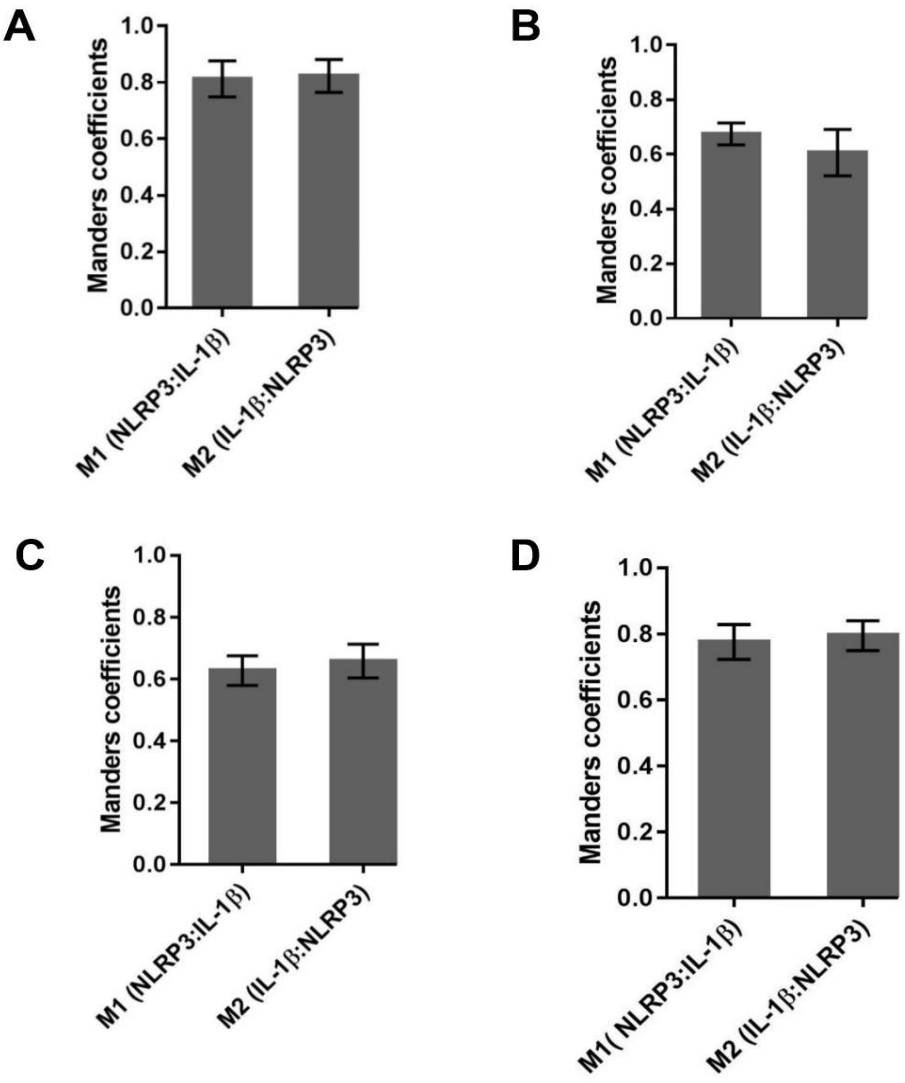


Figure 5-9: Manders colocalisation coefficients (M1 and M2) for **A)** normal colon, **B)** active UC, **C)** active colonic CD, **D)** active ileal CD.

Each data set was compiled from lamina propria regions of interest (ROI), from (n=20 images) 400X images, where averages are presented as mean ± standard deviation. The significance threshold was $p<0.05$.

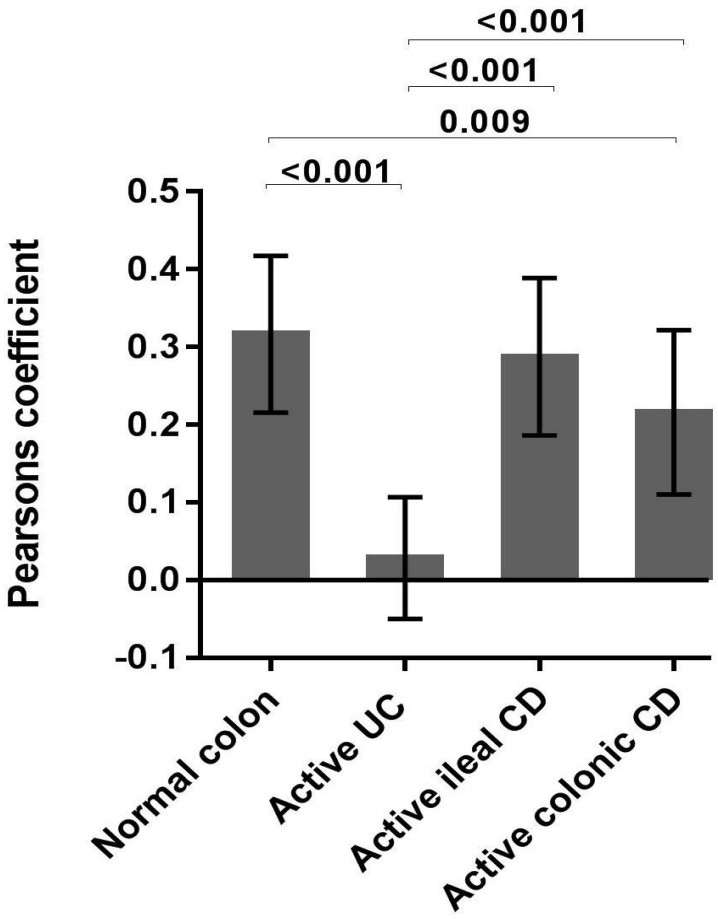


Figure 5-10: Pearson's correlation coefficients for normal colon, active UC and active CD.

Each data set describes the fluorescence intensity distribution between channels and was compiled from lamina propria regions of interest (ROI), from (n=20 images) 400X images, where averages are presented as mean $\pm$ standard deviation. Significance was tested using one way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. The significance threshold was $p<0.05$.

5.3 DISCUSSION

This study describes the colonic localisation of NLRP3 and IL-1β in remission and active IBD and provides evidence that suggests both NLRP3 inflammasome-dependent and independent processes contribute to IL-1β production.

In normal colon, IL-1β was localised to the epithelial layer and generally absent from the lamina propria immune cells, while NLRP3 expression was generally present at low levels in the lamina propria immune cells. With disease activity, the expression of IL-1β unexpectedly shifted from the epithelial cell layer to the lamina propria cells and the expression of both NLRP3 and IL-1β in the lamina propria immune cells intensified. Previously, NLRP3 expression has been demonstrated in granulocytes, such as neutrophils, dendritic cell, monocytes, epithelial cells, T cells and B cells, with subcellular distribution localisation mainly in the cytoplasm [268].

Microscopic architectural abnormalities, inflammatory changes and the influx of lamina propria immune cells are important features for discriminating normal mucosa from IBD [271]. During active UC, neutrophils dominate the lamina propria immune cell population and are often the effector cells surrounding epithelial damage or mucosal inflammation [272]. Consequently, many grading scales for histological assessment of inflammation in UC include the presence and locality of neutrophils [273-275]. Furthermore, the use of neutrophils as a marker of disease activity in UC is supported by leucocyte scanning studies [276, 277].

The lack of acceptable sensitivity, specificity, and reproducibility discourages diagnosis of CD based on the lamina propria infiltrate. Diagnosis of CD therefore relies on the presence of epithelioid granuloma, relatively unchanged crypts or

segmented distribution of crypt atrophy, crypt distortion together with discontinuous focal or patchy inflammation (skip lesions) and mucin preservation in the epithelium at an ulcer edge. Focal inflammation is often characterised by a small collection of inflammatory cells in otherwise normal mucosa [16, 17, 278]. Microscopic structural abnormalities and the influx of lamina propria immune cells are not features of normal mucosa.

Immunofluorescence confocal microscopy demonstrated a high proportion of colocalisation between NLRP3 and IL-1β in normal mucosa however, in active UC, the contribution of NLRP3 to the overall production of bioactive IL-1β was reduced. Together, these results suggest that during normal gut homeostasis inflammasome-dependent caspase-1 is a major contributor to IL-1β production. In contrast, during inflammatory conditions serine proteases produced by the influx of neutrophils into the lamina propria are possibly the main source of bioactive IL-1β and caspase-1 has only a minor role.

Inflammasome-independent processing of proIL-1β in acute inflammatory conditions is well established and has been shown to occur where neutrophils dominate the lamina propria cell populations. For instance, host resistance to disseminated candidiasis is provided by neutrophil derived proteinase 3 activation of IL-1β and not caspase-1 activation [267, 279, 280]. Similarly, during acute arthritis, neutrophil derived proteinase 3 plays a dominant role in in the production of bioactive IL-1β [281]. Again, serine proteases from neutrophils drive the inflammation and production of mature IL-1β in a murine model of osteomyelitis [282, 283].

The administration of IL-1 blocking agents such as rilonacept (Arcalyst, Regeneron, Tarrytown, NY, USA), canakinumab (Ilaris, Novartis, Basel,

Switzerland) and anakinra (Kineret, Amgen, Thousand Oaks, CA, USA) [151] have resulted in remarkable clinical outcomes for patients with hereditary periodic fever syndromes known Cryopyrin-associated periodic syndromes (CAPS). CAPS are a family of autosomal dominant diseases including, familial cold auto-inflammatory syndrome (FCAS), Muckle-Wells syndrome, (MWS), and chronic infantile neurological, cutaneous and articular, (CINCA) syndrome and are associated with the gain of function in NLRP3, which causes continuous IL-1β secretion in the absence of an antagonist. The clinical severity of CAPs varies but in general all syndromes are characterised by recurrent fever, urticarial-like rashes and systemic inflammation [151]. More than 90 disease associated genetic variants of the *NLRP3* gene have been identified for CAPS, the majority are autosomal dominant missense point mutations, located in exon 3, which encodes the NACHT domain [152].

The blockade of NLRP3 using selective small-molecule inhibitors has recently been the focus of much research. MCC950 has been shown to inhibit canonical and non-canonical NLRP3 inflammasome activation and reduce IL-1β production [284]. However based on the results from Chapter 5, in diseases such as UC where neutrophils dominate the lamina propria infiltrate blocking NLRP3 or caspase-1 may not reduce *in vivo* IL-1β levels. Interestingly, in a murine model of arthritis dual blockage of both caspase-1 and proteinase 3 is seen as a potential anti-inflammatory therapeutic option [281].

Taken altogether, this study provides evidence for the inflammasome-dependent and independent processing of IL-1β during active UC. Future IBD research now needs to focus on assessing neutrophil derived IL-1β production and the

therapeutic potential of blocking both caspase-1 and neutrophil derived serine proteases in active UC.

CHAPTER 6 COLONIC LOCALISATION OF NLRP6 AND INTERACTION WITH MUC2 AND E-CADHERIN

6.1 INTRODUCTION

The Nod-like receptor pyrin-containing protein 6 (NLRP6), also known as PYPAF5, is a member of the nucleotide-binding oligomerisation domain (NOD)-containing protein receptor (NLR) family for which there are 23 human and 34 mouse members [20, 70]. Evidence for NLRP6 inflammasome formation has been provided by *in vitro* work demonstrating the colocalisation of NLRP6 with ASC in the cytoplasm and the production of IL-1β [285]. In the murine system, NLRP6 has been shown to regulate goblet cell mucin production and secretion, regulate self-renewal and proliferation [226], protect against chemical induced intestinal injury and tumorigenesis [129, 186, 286] and negatively regulate inflammasome signalling [130]. Despite all the proposed functions for NLRP6 the activating ligand still remains unknown.

Epithelial cells are protected from bacterial, chemical and physical insults by a stratified layer of mucus. The main protein component of the mucus barrier, mucin 2 (MUC2) is produced within specialised goblet cells where it is stored within a highly organised array of vertically orientated microtubules and intermediate filaments known as the theca. The theca defines the outer limit of the storage granular zone and separates granules from the remaining cytoplasm [287]. Evidence for the modulation of Muc2 production by the microbial ecology is provided firstly, by the reduction in goblet cell number and decreased rates of epithelial cell turnover in mice grown in germ-free conditions [1, 288] and

secondly, by the ability of bacterial ligands to induce Muc2 production in intestinal explant cultures [289].

Intestinal goblet cells are functionally heterogeneous and have been shown to contribute to the regulation of innate immune defences [289-291]. Goblet cell associated antigen passages (GAP) cells endocytose soluble material from the intestinal lumen and are capable of translocating the antigen to underlying CX_3CR_1 positive dendritic cells which then migrate to draining lymph nodes [291-293].

Work by Birchenough et al [289] in murine explants have demonstrated that TLR ligands, LPS, its subcomponent lipid A, the synthetic triacylated lipopeptide P3CSK4, flagellin and the neurotransmitter acetylcholine analog carbachol (Cch) induce Muc2 secretion from goblet cells. Furthermore, lower crypt goblet cells were shown to secrete Muc2 in response to Cch but not TLRs while a sentinel goblet cell (SenGC) localised to the colonic crypt entrance endocytosed responsive TLRs but not Cch and was expelled from the epithelium. The expulsion of SenGCs was shown to be driven by NLRP6 inflammasome activation since intrarectal P3CSK4 treatment resulted in endocytosis of P3CSK4 but not expulsion of SenGCs. The expelled SenGC triggered an intercellular signal via gap junctions and increased cytoplasmic Ca^{2+} that induced Muc2 secretion in adjacent responsive goblet cells. Compound exocytosis and cascading Muc2 secretion represents a mechanism that shifts bacterial pathogens and prevents colonisation of intestinal crypts. In contrast, work by Wlodarska et al [187] found that genetic deletion of *Nlrp6, Asc* and *Casp1/11* was associated with a diminish intact mucus layer, goblet cell hyperplasia and abrogated mucin secretion suggesting defects in NLRP6 affect autophagy and mucin release. Interestingly,

the lack of an intact mucus layer was not a feature of the inflammasome deficient mice used by Birchenough et al [289].

Determining the role of NLRP6 in the pathogenesis of gastrointestinal diseases has been aided by the use of *Nlrp6* knockout mice and models of chemically induced colitis, however many differences exist between murine colitis and human IBD and many questions still remain unanswered. Based on the available murine Nlrp6 data it is hypothesised that NLRP6 also regulates goblet cell mucin production and secretion in human IBD. Gene expression data presented in Chapter 3 highlighted the disease specific upregulation of *NLRP6* in ileal CD. Following on from these results the aim of Chapter 6 was to examine the cellular localisation of NLRP6, its relationship to MUC2 and E-cadherin, and the impact of NLRP6 on MUC2 expression in human cell lines.

6.2 RESULTS

6.2.1 NLRP6 ANTIBODY OPTIMISATION

In order to investigate the localisation of NLRP6 in colon tissue it was first necessary to establish the efficiency of the NLRP6 antibody (NBP2-31372, Novus Biological, Littleton, CO, USA). Positive cytoplasmic and nuclear staining for NLRP6 was observed in the breast ductal/acinar epithelium of normal human breast tissue (Figures 6-1 and 6-2). This is consistent with the NLRP6 (NBP2-31372), Novus Biological product information sheet (Appendix 7) which also demonstrates positive cytoplasmic and nuclear staining in the ductal region of formalin fixed paraffin embedded human breast tissue using immunohistochemistry analysis.

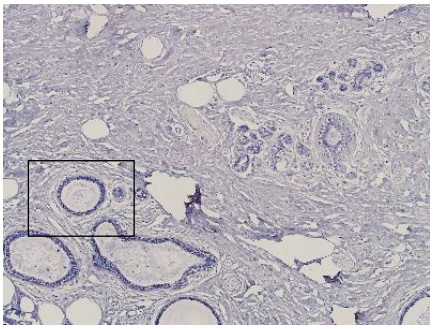
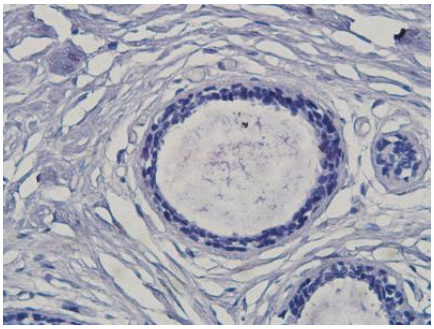
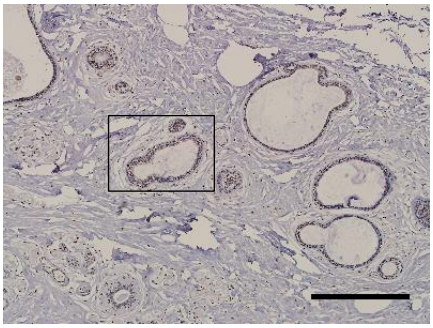
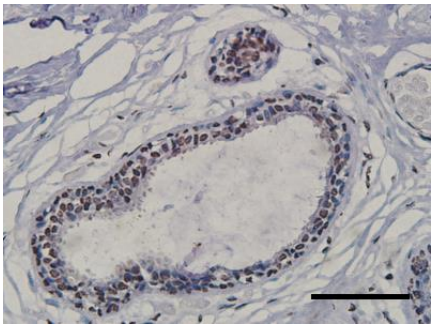
NLRP6	100X	400X
Negative control (human breast)		
Positive control (human breast)		

Figure 6.1 (previous page): Localisation of NLRP6 in normal human breast tissue as analysed by immunohistochemistry.

Tissue was paraffin embedded and cut into 5 µm sections. The NLRP6 antibody (NBP2-31372, Novus Biological, Littleton, CO, USA) was diluted 1:300. Scale bars represent 200 µm for 100X and 50 µm for 400X magnification.

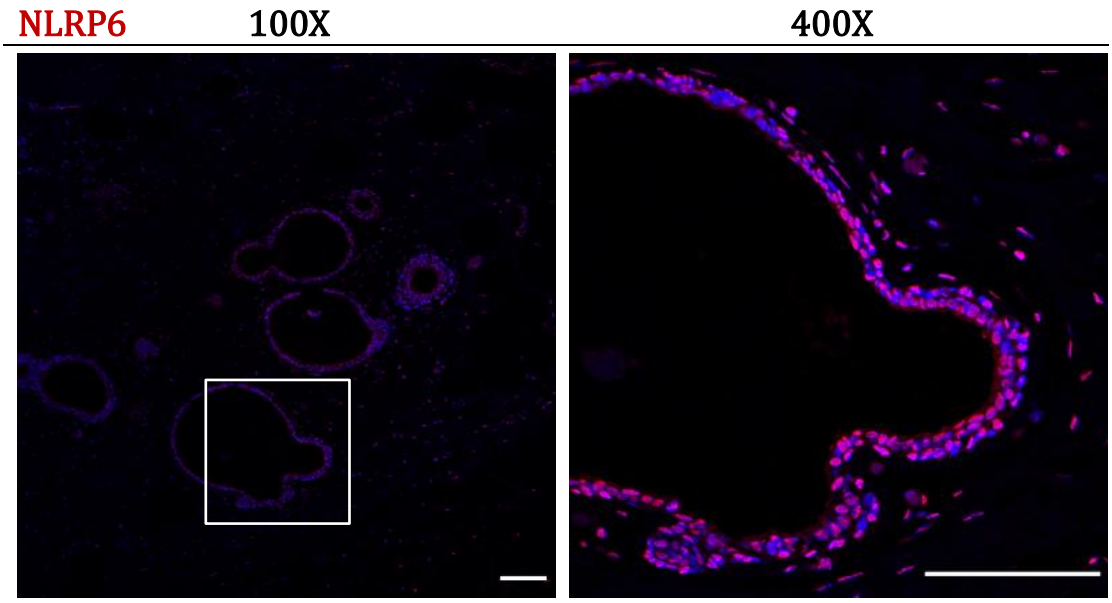


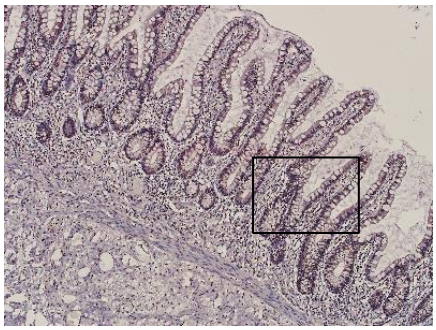
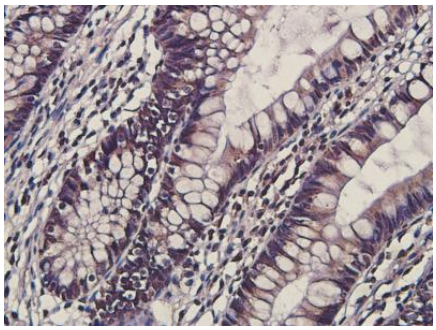
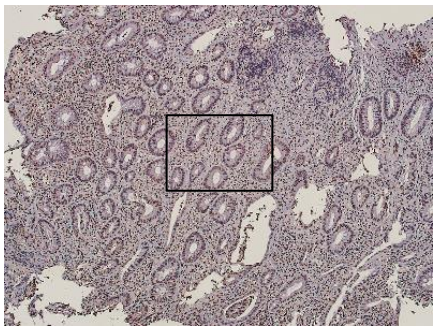
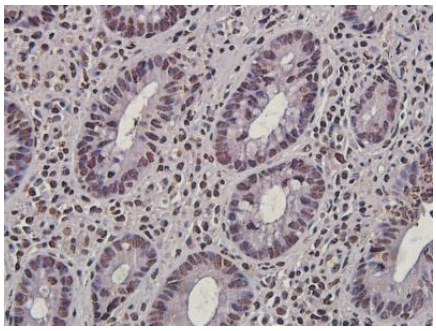
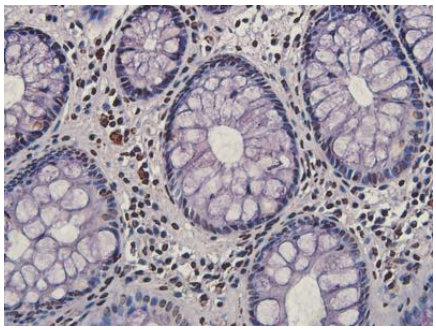
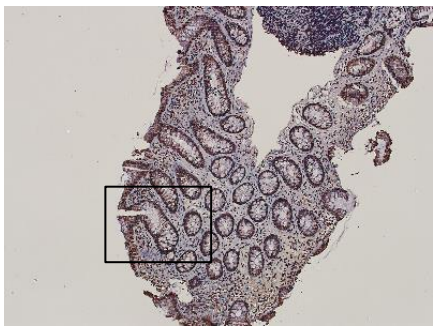
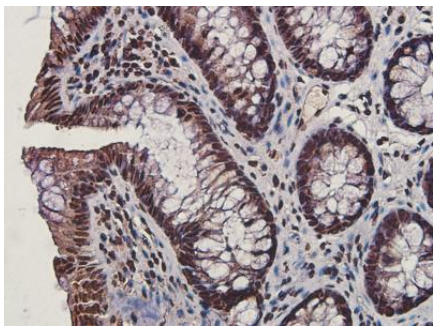
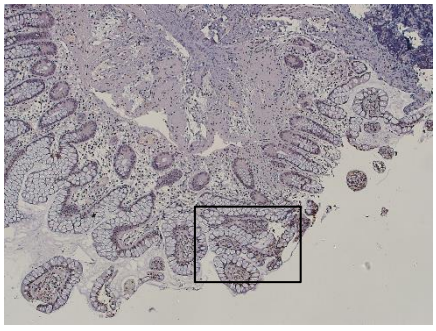
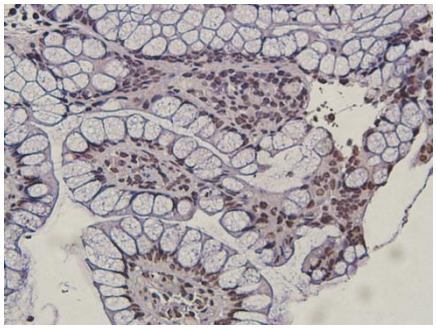
Figure 6-2: Representative immunofluorescence confocal images of NLRP6 localisation in normal human breast tissue

All sections were paraffin embedded, cut into 5 µm sections and mounted on superfrost plus slides. Localisation of NLRP6 (NBP2-31372, Novus Biological, Littleton, CO, USA) in normal human breast diluted 1:100 and visualised using Alexa Fluor®647 conjugated goat anti-rabbit IgG (red). Nuclei were stained with 4' ,6 diamidino-2-phenylindole (DAPI, blue). Scale bars represent 100 µm for both the 100X and 400X magnification.

6.2.2 COLONIC LOCALISATION OF NLRP6

Immunohistochemistry and immunofluorescence confocal microscopy were used to determine the colonic localisation of NLRP6. Prominent NLRP6 expression was observed in the colon and ileum biopsies from active ileal CD patients. The high expression of NLRP6 was almost exclusively localised to the epithelial cell layer, including myofibroblasts, however a moderate number of neutrophils and monocytic linage cells residing in the lamina propria also exhibited high NLRP6 expression (Figures 6-3 and 6-4).

In both, active colonic CD and remission CD, staining of the epithelial cell layer was unremarkable and only scattered NLRP6 expression was noted within the lamina propria immune cells. In the normal colon, moderate cytoplasmic NLRP6 expression was present in the epithelial cell layer and only scattered staining was evident in lamina propria cells. In contrast, no cytoplasmic epithelial NLRP6 expression was evident in active UC or remission UC. However, in active UC a moderate number of lamina propria cells expressed NLRP6 while in UC remission the expression was minimal. Quantitative analysis of NLRP6 immunohistochemistry staining confirmed the high NLRP6 expression in ileal CD (active ileal CD *vs* active colonic CD, $p<0.001$; active ileal CD *vs* remission CD, $p<0.001$) (Figure 6-7A).

NLRP6	100X	400X
Normal colon		
Active UC		
Remission UC		
Active ileal CD		
Active ileal CD (ileum biopsy)		

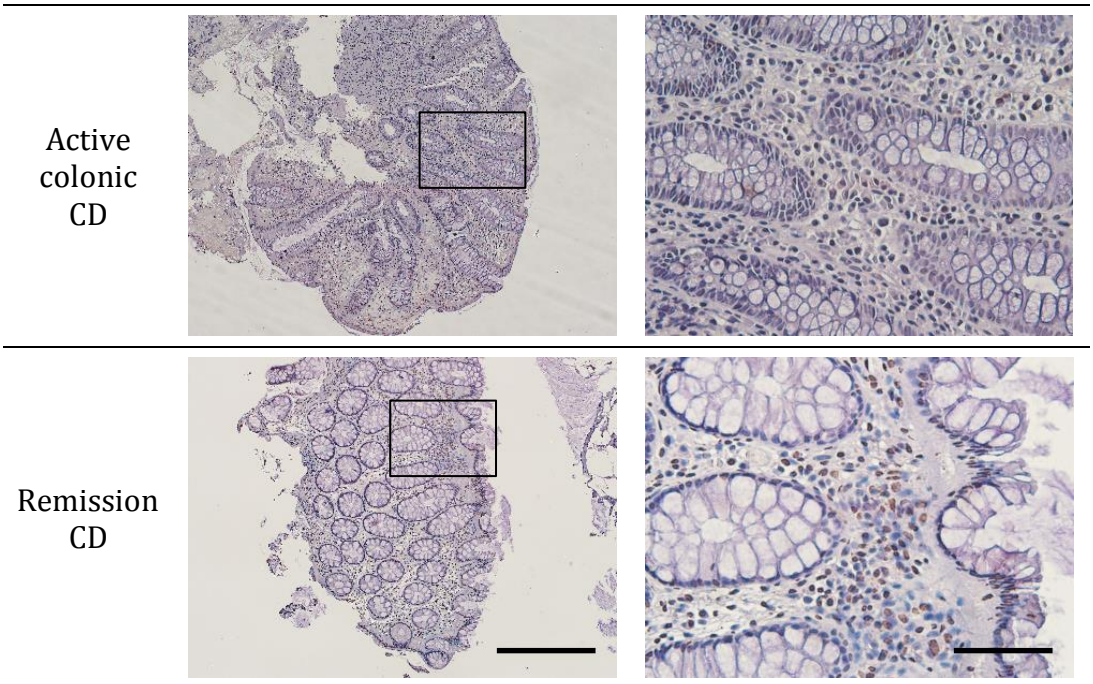
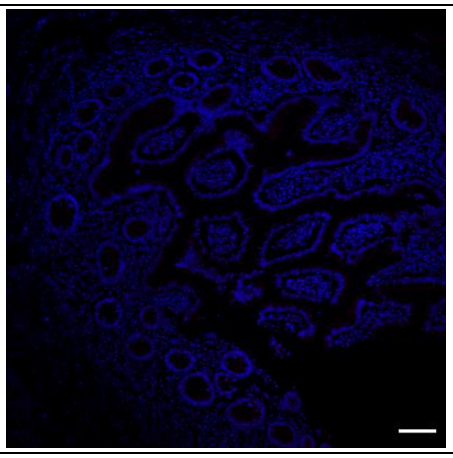
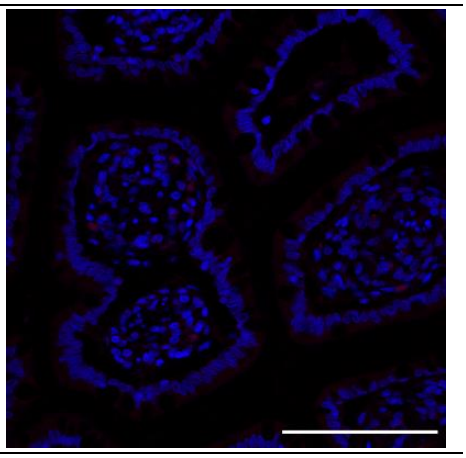
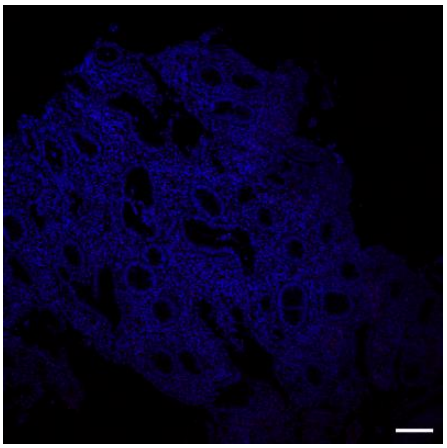
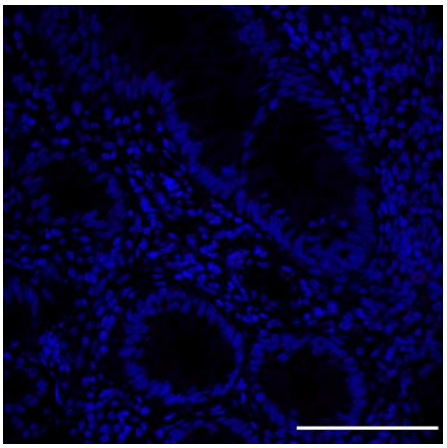
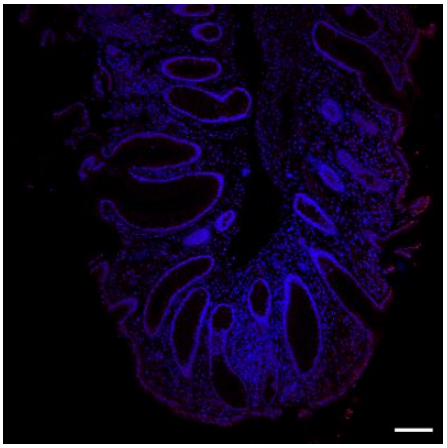
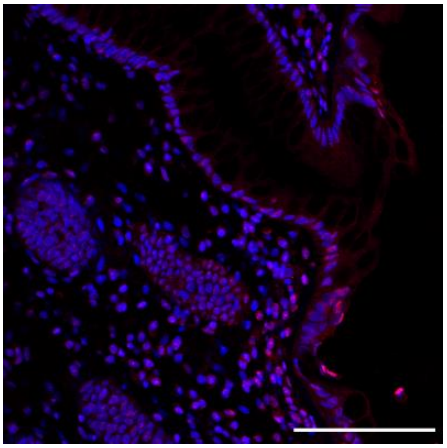
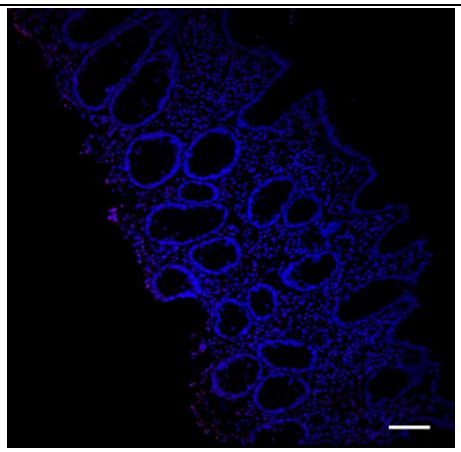
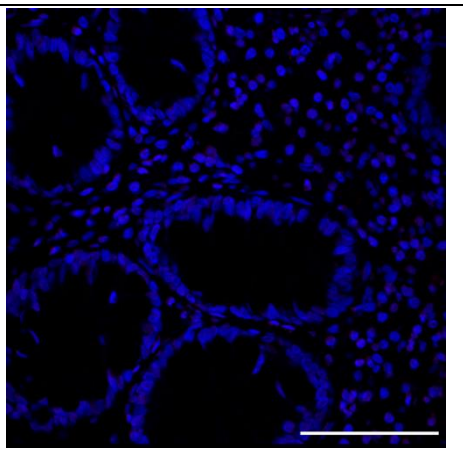


Figure 6-3: Representative immunohistochemistry images of NLRP6 expression in normal colon, active UC, remission UC, active ileal and colonic CD and remission CD.

All biopsies were taken from the left colon (unless stated as ileum biopsy), paraffin embedded, cut into 5 µm sections and incubated with NLRP6 (NBP2-31372, Novus Biological, Littleton, CO, USA) at a dilution of 1:200. Scale bars represent 200 µm for 100X and 50 µm for 400X magnification.

Figure 6-4 (next page): Representative immunofluorescence images of NLRP6 localisation in normal colon, active UC, active ileal and colonic CD.

All biopsies were taken from the left colon, paraffin embedded, cut into 5 µm sections and incubated with NLRP6 (NBP2-31372, Novus Biological, Littleton, CO, USA, 1:100 dilution) and visualised using Alexa Fluor®647 conjugated goat anti-rabbit IgG (red). Nuclei were stained with 4' ,6 diamidino-2-phenylindole (DAPI, blue). Scale bars represent 100 µm for both the 100X and 400X magnification.

NLRP6	100X	400X
Normal colon		
Active UC		
Active ileal CD		
Active colonic CD		

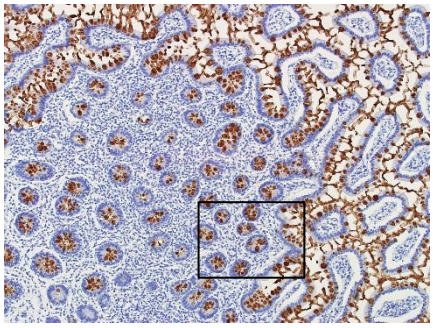
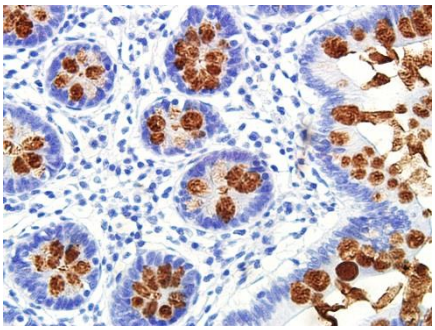
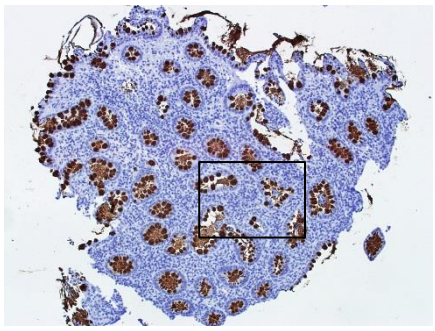
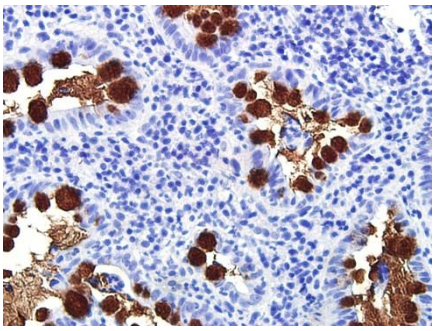
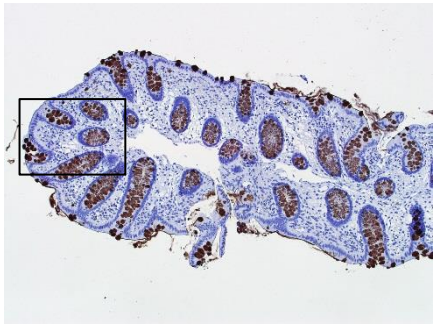
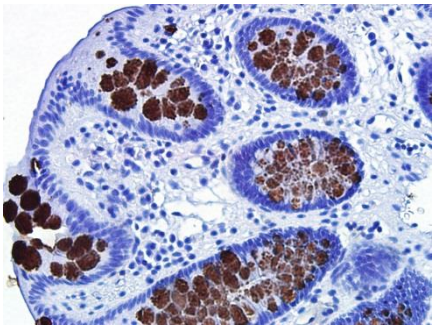
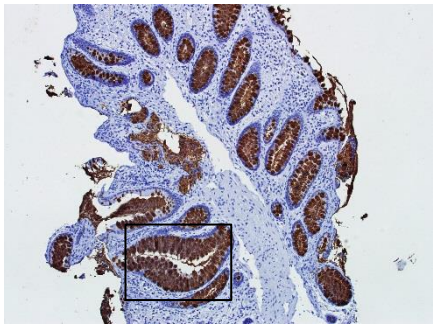
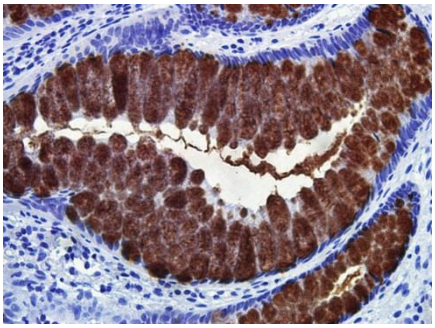
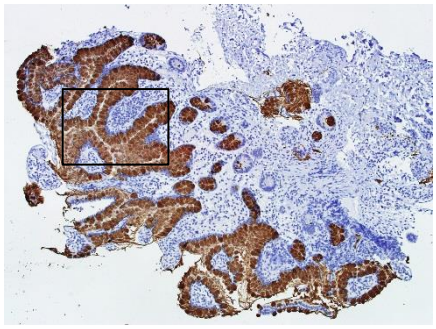
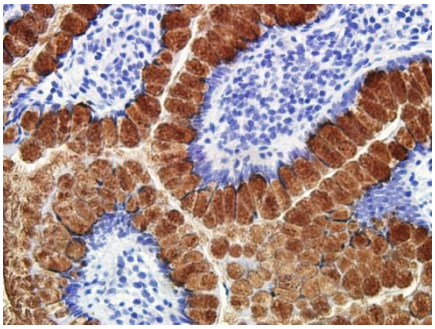
6.2.3 MUC2 ANTIBODY EFFICIENCY AND COLONIC LOCALISATION

Immunohistochemistry and immunofluorescence confocal microscopy was used to determine the colonic localisation of MUC2. The efficiency of the MUC2 antibody (H:300: sc15334, Santa Cruz, Dallas, Texas, USA) was confirmed by the consistent staining of goblet cells theca contents across all colon biopsy samples (Figures 6-5 and 6-6).

The expression of MUC2 was found to be highly variable in distribution and depth of staining across all IBD phenotypes (Figures 6-5 and 6-6). In remission and active CD the architecture and packaging of MUC2 granules was highly organised and this structural organisation was lacking in active UC and in the normal colon (Figure 6-5).

Normally colonic crypts contain smaller goblet cell theca containing less MUC2 in the lower crypts and larger MUC2 filled theca in the upper crypt [289]. Surprisingly, in active ileal CD, active colonic CD and remission CD large tightly packed theca containing MUC2 granules were observed along the entire crypt length and this overcrowding of goblet cells was often associated with crypt distortion.

Quantitatively, the expression of MUC2 in active ileal CD was greater than in active UC (active ileal CD vs active UC, $p=0.002$) and the expression in both UC remission and active UC was less than that observed in the normal colon (normal *vs* remission UC, $p<0.001$; active UC *vs* remission UC, $p=0.002$). Overall, the active tissues of both UC and CD showed a higher variability in MUC2 expression as indicated by the increased standard deviation error bars (Figure 6-7B).

MUC2	100X	400X
Normal colon		
Active UC		
Remission UC		
Active ileal CD		
Active ileal CD (ileum biopsy)		

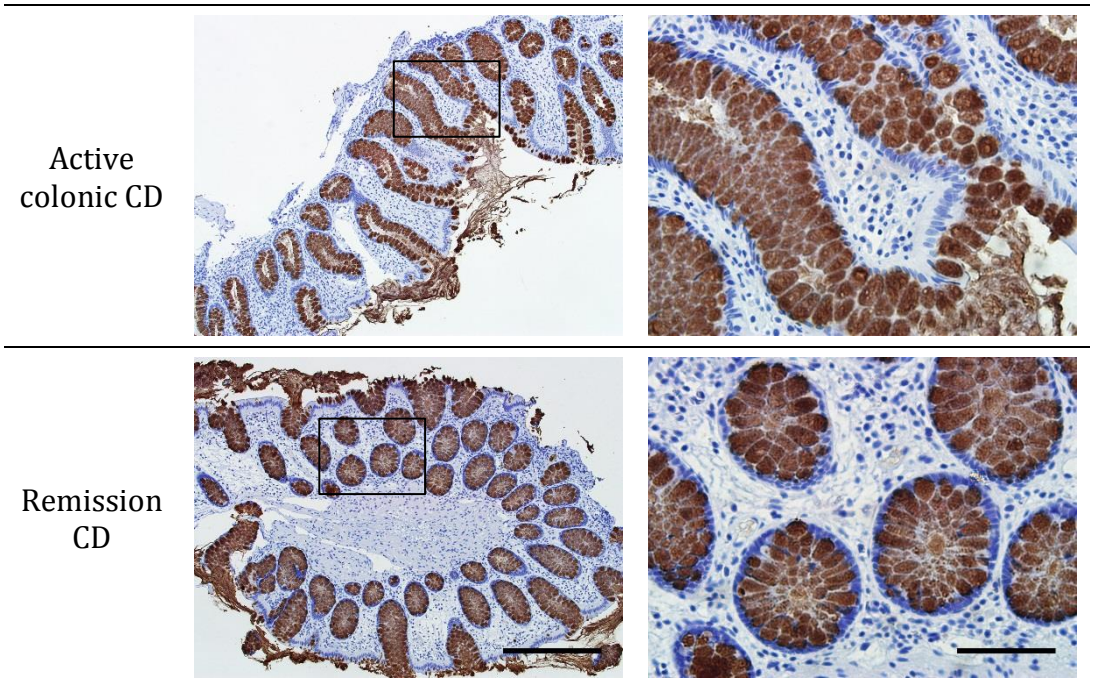
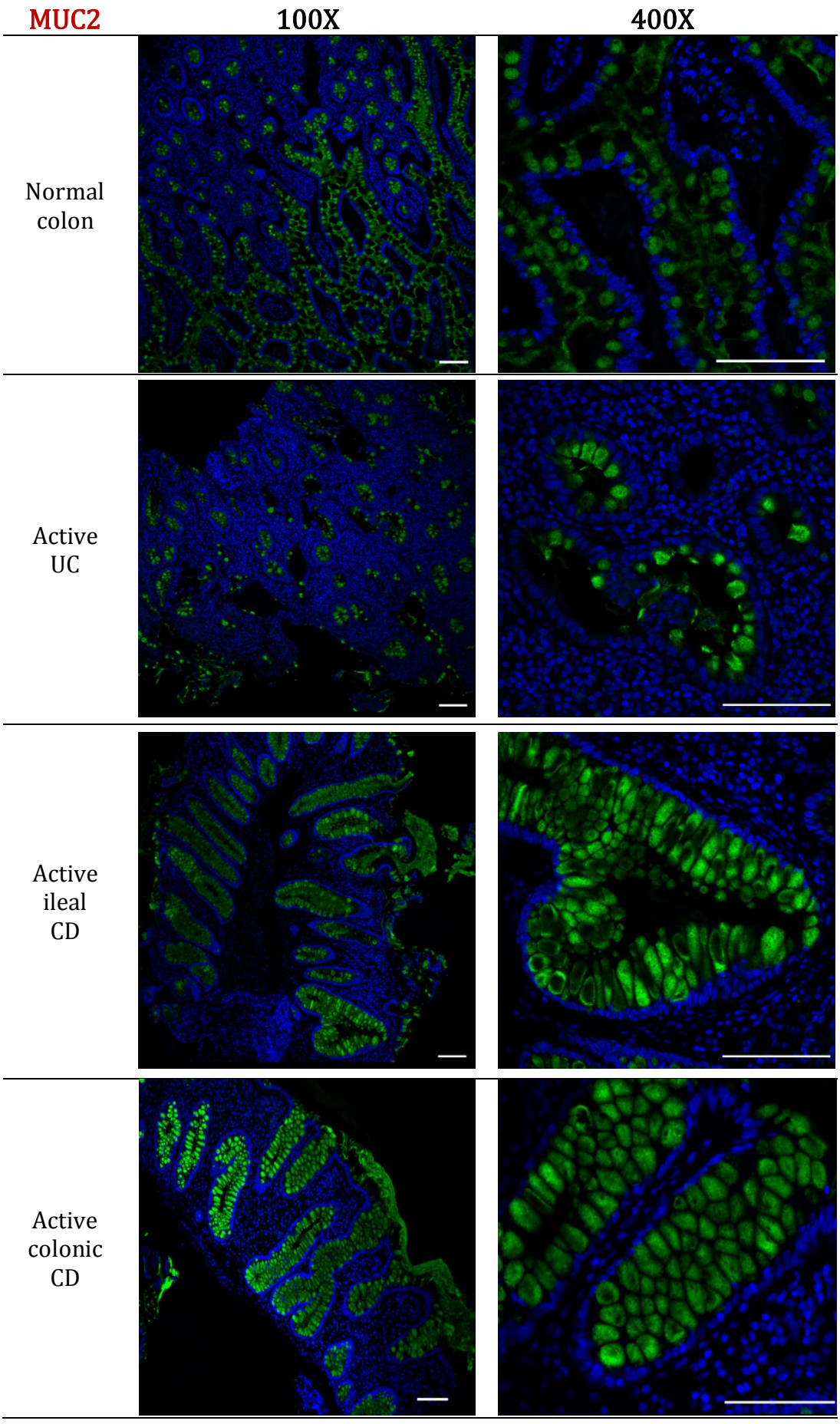


Figure 6-5: Representative immunohistochemistry images of MUC2 expression in normal colon, active UC, remission UC, active ileal and colonic CD and remission CD.

All biopsies were taken from the left colon (unless stated as ileum biopsy), paraffin embedded, cut into 5 µm sections and incubated with a MUC2 rabbit polyclonal antibody (H:300: sc15334, Santa Cruz, Dallas, Texas, USA), at a dilution of 1:300. Scale bars represent 200 µm for 100X and 50 µm for 400X magnification.

Figure 6-6 (next page): Representative immunofluorescence images of MUC2 localisation in normal colon, active UC, active ileal and colonic CD.

All biopsies were taken from the left colon, paraffin embedded, cut into 5 µm sections and incubated with MUC2 (H:300: sc15334, Santa Cruz, Dallas, Texas, USA, 1:200 dilution) and visualised using Alexa Fluor®647 conjugated goat anti-rabbit IgG (green). Nuclei were stained with 4' ,6 diamidino-2-phenylindole (DAPI, blue). Scale bars represent 100 µm for both the 100X and 400X magnification.


MUC2
100X
400X
Normal colon
Active UC
Active ileal CD
Active colonic CD

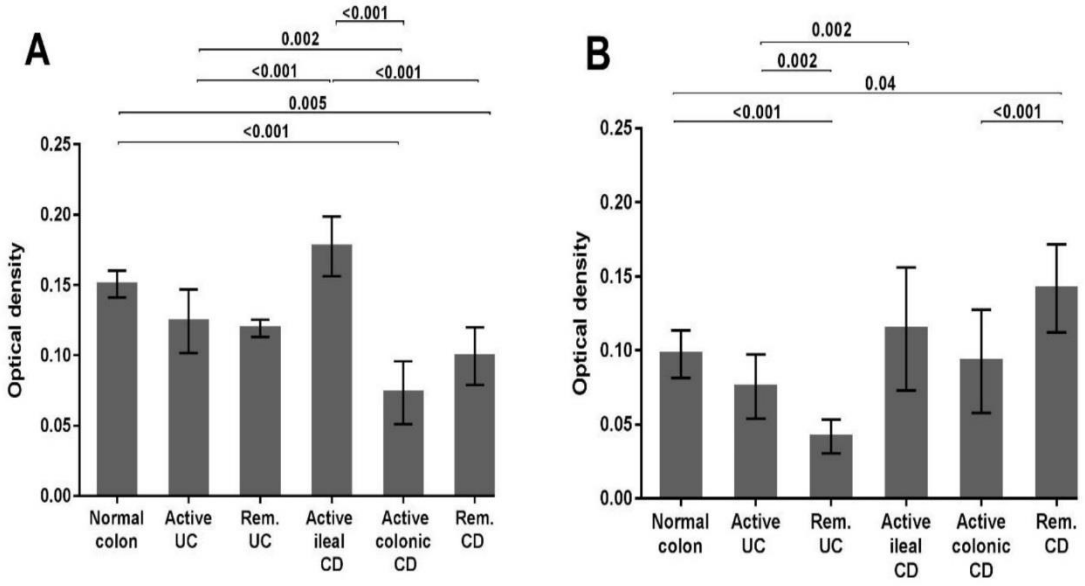


Figure 6-7: Quantification of NLRP6 and MUC2 immunohistochemistry staining in biopsy sections from normal and IBD patients

A) Quantitative analysis of NLRP6 expression in sections from normal colon (total number of images analysed=18), active UC (n=43), remission UC (Rem. UC, n=20, active ileal CD (n=34), active colonic CD (n=18) and remission CD (Rem. CD, n=12). **B)** Quantitative analysis of MUC2 expression in sections from normal colon (n=20), active UC (n=62), remission UC (Rem. UC, n=20), active ileal CD (n=24), active colonic CD (n=30) and remission CD (Rem. CD, n=30).

Paraffin embedded left colon mucosal biopsies were analysed by immunohistochemistry and the optical intensity of DAB staining due to MUC2 was determined using FIJI software. All data are presented as mean $\pm$ standard deviation. Statistical significance was evaluated using Dunn's multiple comparison one-way analysis of variance (ANOVA). The significance threshold was $p<0.05$.

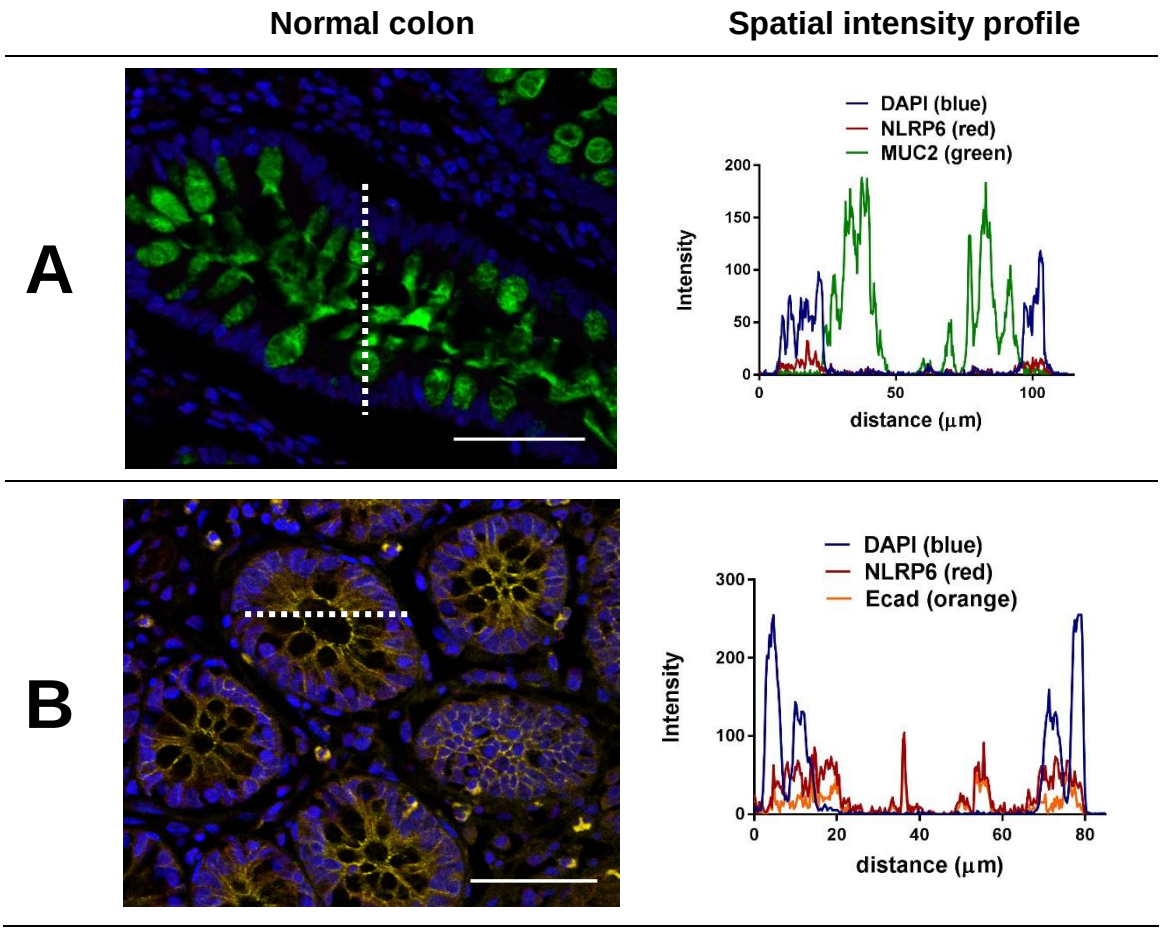


Figure 6-8: Representative colocalisation images of NLRP6, MUC2 and E-cadherin in normal colon biopsies.

A) Parrafin embedded normal colon biopsies were stimultaneously stained with NLRP6 (red) (NBP2-31372, Novus Biological, Littleton, CO, USA) and MUC2 (green) (F-2: sc-515032, Santa Cruz Biotechnology, Dallas, Texas, USA) and visualised using AexaFluor® 647 conjugated rabbit anti-goat and Alexa Fluor®555 conjugated mouse anti-goat respectively. **B)** Parrafin embedded normal colon biopsies were stimultaneously stained with NLRP6 (red) (NBP2-31372, Novus Biologicals, Littleton, CO, USA) and E-cadherin (yellow) (NCH-38;M2612, Dako North America, Carpinteria, CA, USA) and visualised using AexaFluor®647 conjugated rabbit anti-goat and Alexa Fluor®555 conjugated mouse anti-goat respectively. Nuclei were stained with 4' ,6 diamidino-2-phenylindole (DAPI, blue). Spatial intensity profile is indicated by the dotted line. Images are 400X and scale bars = 50 μm.

6.2.4 COLOCALISATION OF NLRP6 WITH MUC2 AND E-CADHERIN

To determine the spatial relationship of NLRP6 to the major mucin protein, MUC2 and the epithelial cell marker, E-cadherin, biopsies were double stained for NLRP6 and MUC2 or NLRP6 and E-cadherin and examined using immunofluorescence confocal microscopy. Using visual inspection and spatial profiling there was no evidence of NLRP6 colocalising with MUC2 in active ileal CD biopsies (Figure 6-9A) however strong colocalisation was evident with E-cadherin (Figures 6-9B and 6-9C). Previously, losses in E-cadherin have been associated with active disease processes [294], however this was not observed in active ileal CD. Additionally, in ileal CD a high proportion of upper goblet cells cell exhibited high cytoplasmic NLRP6 expression were observed while other goblet cells remained NLRP6 negative (Figure 6-9A). In accordance with mouse studies by Birchenough et al [289] it is proposed that the high NLRP6 expressing goblet cells would be eventually expelled from the epithelium in a NLRP6 dependent manner. In the normal colon biopsies, NLRP6 was found to colocalise with E-cadherin and not MUC2 and the apical goblet cell specific expression of NLRP6 was not evident (Figure 6-8).

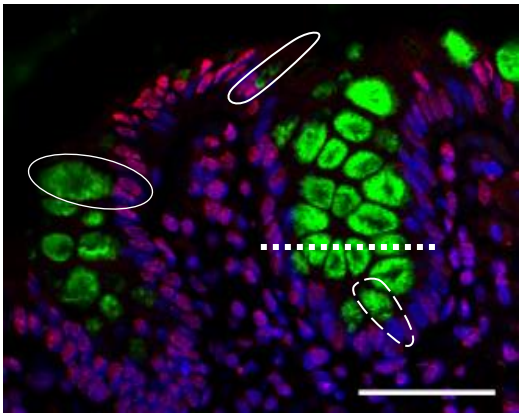
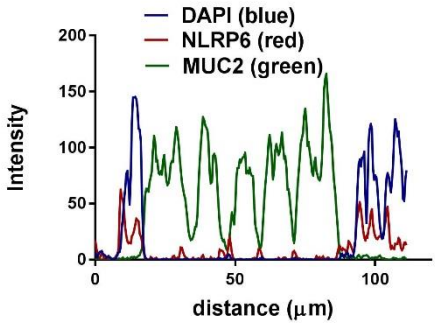
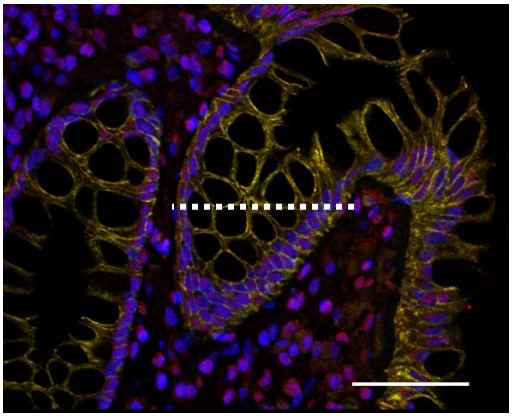
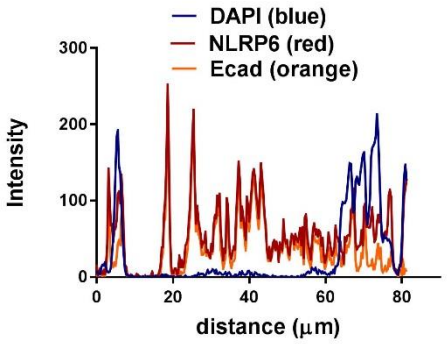
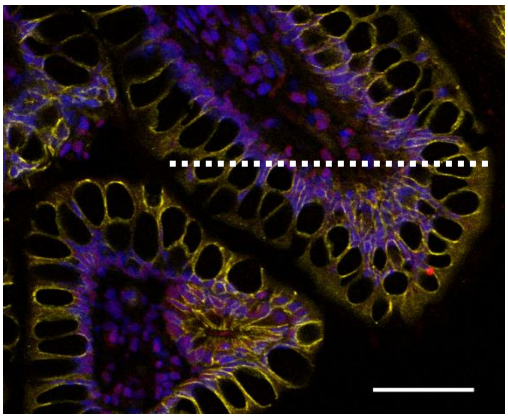
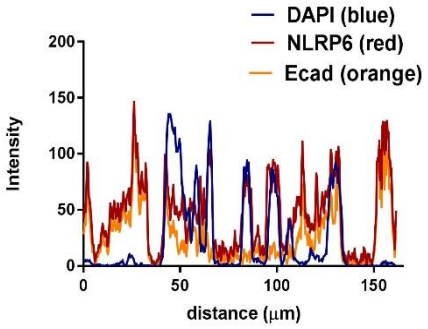
	Active ileal CD	Spatial intensity profile
A		
B		
C		

Figure 6-9 (previous page): Representative colocalisation images of NLRP6, MUC2 and E-cadherin in ileal CD biopsies.

A) Parrafin embedded active ileal CD (left colon) biopsies) were stimultaneously stained with NLRP6 (red) (NBP2-31372, Novus Biological, Littleton, CO, USA) and MUC2 (green) (F-2: sc-515032, Santa Cruz Biotechnology, Dallas, Texas, USA) and visualised using AexaFluor®647 conjugated rabbit anti-goat and Alexa Fluor®555 conjugated mouse anti-goat respectively. Solid lines indicates goblet cell with high NLRP6 expression. Dashed line indicates goblet cells with no NLRP6 expression. **B)** Parrafin embedded active ileal CD (left colon) biopsies were stimultaneously stained with NLRP6 (red) (NBP2-31372, Novus Biologicals, Littleton, CO, USA) and E-cadherin (yellow) (NCH-38;M2612, Dako North America, Carpinteria, CA, USA) and visualised using AexaFluor®647 conjugated rabbit anti-goat and Alexa Fluor®555 conjugated mouse anti-goat respectively.

C) Parrafin embedded active ileal CD (ileum) biopsies were stimultaneously stained with NLRP6 (red) (NBP2-31372, Novus Biologicals, Littleton, CO, USA) and E-cadherin (yellow) (NCH-38;M2612, Dako North America, Carpinteria, CA, USA) and visualised using AexaFluor®647 conjugated rabbit anti-goat and Alexa Fluor®555 conjugated mouse anti-goat respectively.

Nuclei were stained with 4' ,6 diamidino-2-phenylindole (DAPI, blue). Spatial intensity profile is indicated by the dotted line. Images are 400X and scale bars = 50 μm.

6.2.5 THE INDUCTION OF NLRP6 IN COLONIC CELL LINES

To examine the effect of *NLRP6* on *MUC2* expression, a known PPAR-γ agonist, rosiglitazone, in the absence of LPS priming was used to induce NLRP6 expression in the human cell lines, LS174T and HT29.

In the LS174T cell line, rosiglitazone at concentrations from 0.5-50 μM increased the expression of *NLRP6* ($p=0.01$). The increase in *NLRP6* expression resulted in repression of *MUC2* ($p=0.002$) and *PPAR-γ* expression ($p=0.01$). The expression of *IL-1β* and *IL-18* remained unchanged despite the increase in *NLRP6* expression (Figure 6-10).

For the HT29 cell line, a 6-hour incubation of rosiglitazone at concentration between (1-100 μM) failed to induce *NLRP6* expression. Interestingly, the expression of *MUC2* increased without *NLRP6* induction and the expression of *IL-1β* reduced. The expression of *PPAR-γ* was unaffected by increasing concentrations of rosiglitazone (Figure 6-11).

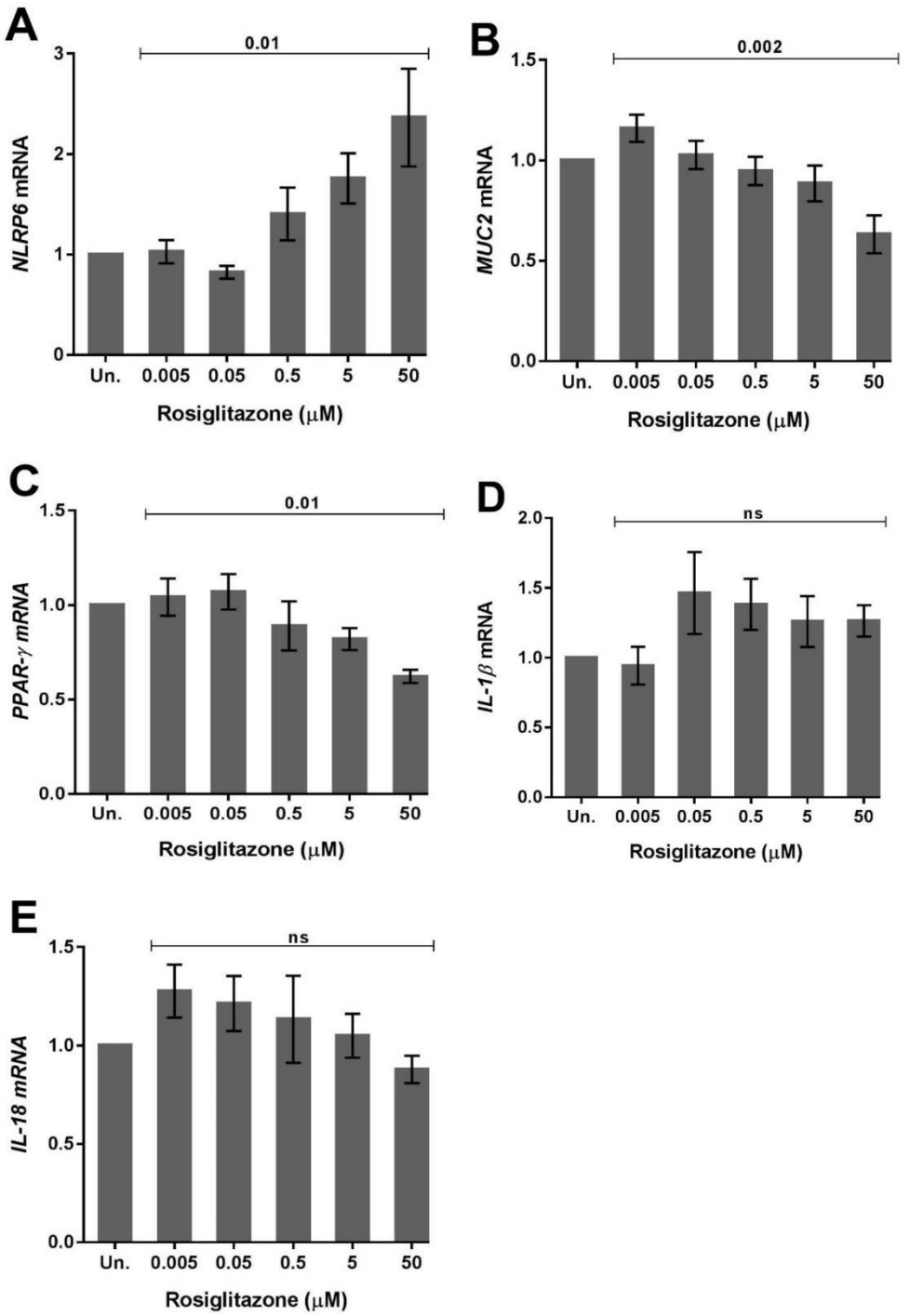


Figure 6-10: mRNA expression of *NLRP6, MUC2, PPAR-γ, IL-1β* and *IL-18* in LS174T cells treated with Rosiglitazone.

Rosiglitazone of varying concentrations (5-50,000 nM) was used to induce NLRP6 expression in HT29 cells. Results are relative to ethanol-treated control cells (Un.=unstimulated) and normalised to the housekeeping gene, EEF2. Data are expressed as the mean ± SEM of 3 independent experiments carried out in duplicate. Differences between group means were assessed using one-way analysis of variance (ANOVA). The significance threshold was $p<0.05$.

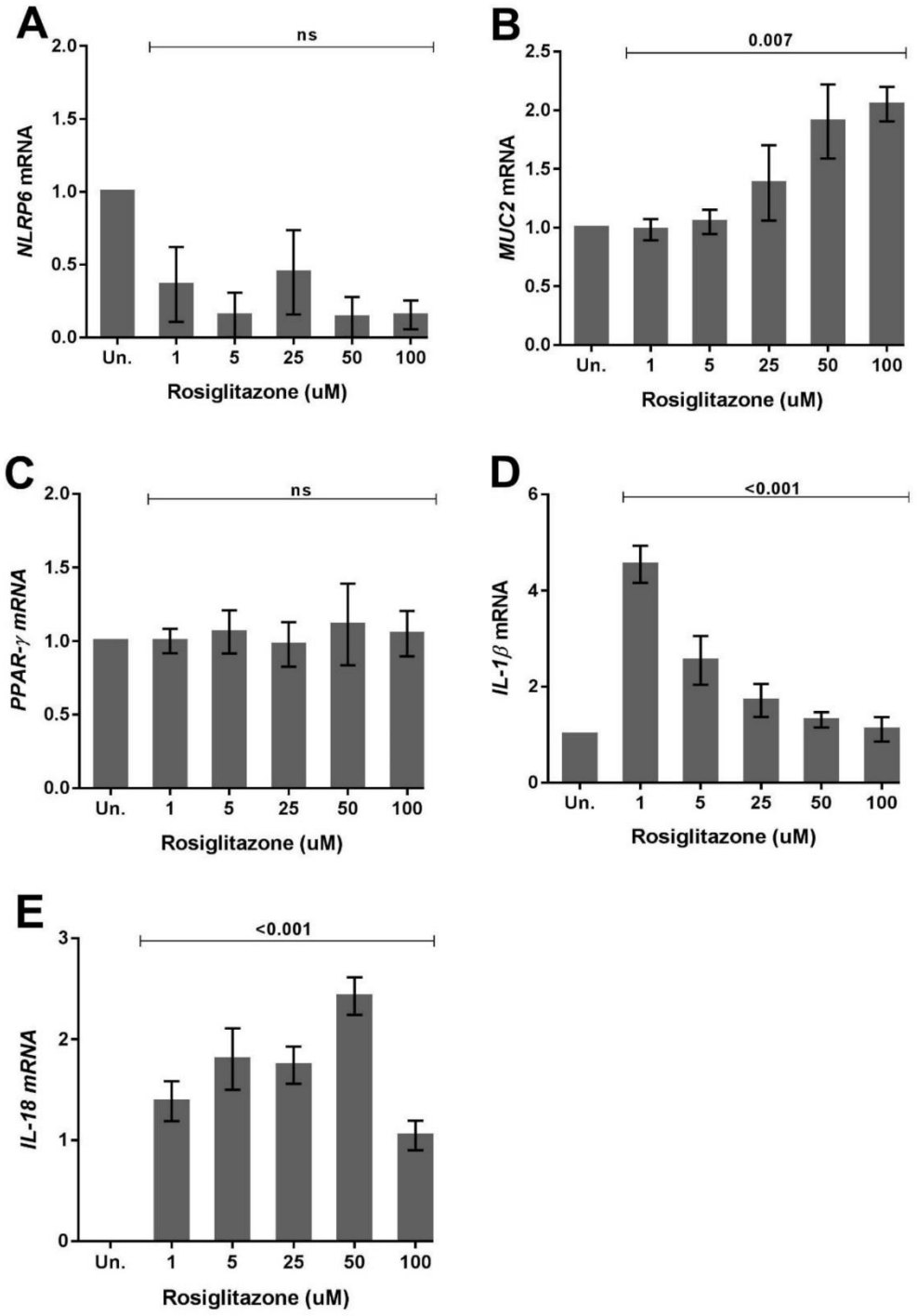


Figure 6-11: mRNA expression of *NLRP6, MUC2, PPAR-γ, IL-1β* and *IL-18* in HT29 cells treated with Rosiglitazone.

Rosiglitazone of varying concentrations (1-100 μM) was used to induce NLRP6 expression in HT29 cells. Results are relative to ethanol-treated control cells (Un.=unstimulated) and normalised to the housekeeping gene, EEF2. Data are expressed as the mean ± SEM of 3 independent experiments carried out in duplicate. Differences between group means were assessed using one-way analysis of variance (ANOVA). The significance threshold was $p<0.05$.

6.3 DISCUSSION

This study describes the colonic localisation of NLRP6 and MUC2 expression in IBD and identifies a NLRP6 expressing goblet cell located predominantly in the upper portion of the intestinal crypt, which suggests a possible role for NLRP6 in goblet cell expulsion.

In colonic and ileum biopsies from active ileal CD, abundant NLRP6 expression was demonstrated in the epithelial cell layer, colonic myofibroblasts and immune cells of the lamina propria. In contrast, NLRP6 in UC was localised to the influx of immune cell within the lamina propria and absent from the epithelial cell layer. Murine Nlrp6 is highly expressed in both the small and large intestine [129, 226] including the intestinal epithelial cells [129, 186, 226], colonic myofibroblasts [226], granulocytes, T cells and macrophages [286] but is reduced in mice adenocarcinoma [226]. Expression data for NLRP6 in humans is limited, however RNA sequencing and antibody-based profiling of non-diseased human gut tissue have defined NLRP6 expression to be restricted to the duodenum, jejunum/ileum [295] and absent from the colon [295, 296].

Colonic myofibroblasts are a major source of modulators of the Wnt signalling pathway which governs approximately 80 genes involved in the differentiation, proliferation and upward migration of gastrointestinal epithelial cells. In mice, the presence of NLRP6 expression in colonic myofibroblasts has previously been disputed however the results of this study are consistent with Normand et al [226].

NLRP6 was localised to the goblet cell cytoplasm and did not colocalise with mucin 2 granules within the theca. Intestinal goblet cells have been shown to be functionally heterogeneous [289-291]. Work by Birchenough et al [289]

proposed that the upper crypt, apical orientated goblet cells are similar to SenGC and NLRP6 directs MUC2 release and goblet cell expulsion. In accordance, this study also identified an upper crypt NLRP6 expressing goblet cell in active ileal CD, suggesting the possibility of a NLRP6 driven goblet cell release. The compound exocytosis of goblet cells and cascading MUC2 secretion are thought to be a mechanism for shifting bacteria away from the crypt opening. In contrast to Birchenough et al [289], many high-NLRP6 expressing goblet cells were present along the crypt length suggesting constant goblet cell expulsion and MUC2 secretion in active ileal CD. Interestingly, CD is often associated with a thickened mucus layer that is constantly being secreted while in UC the mucus layer is thinned which fails to prevent penetration by pathogens [11, 297].

The induction of *NLRP6* using rosiglitazone failed to increase the expression of *MUC2* in LS174T cells. Rosiglitazone is a PPAR-γ synthetic agonist and in the colon has the potential to exert an anti-inflammatory effect [298]. The repression of *MUC2* could therefore be due to minimal bacterial activity and unrelated to NLRP6 activity. Similarly, the increased *MUC2* expression in HT29 cells was probably not a result of NLRP6 activity since rosiglitazone failed to induce *NLRP6*.

NLRP6 was found to colocalise with E-cadherin in colon and ileum biopsies from ileal CD patients. E-cadherin is the major constituent of adherens junctions and is required for the maintenance of architecture, cellular integrity, mediation of intercellular associations and cell polarisation [299, 300]. Cellular adhesion, maturation and the correct placement of paneth and goblet cells is dependent on the proper expression of E-cadherin [300]. E-cadherin has been shown to recruit Ras-related C3 botulinum toxin substrate 1 (Rac1) which anchors the E-cadherin-catenin complex to actin thereby stabilising the cellular structure [301].

Previously, only transient losses of E-cadherin have been associated with active CD, with more pronounced losses being seen in active UC [294, 302, 303]. Given the high expression of NLRP6 in colonic myofibroblasts and the tight association with E-cadherin, one can speculate that NLRP6 may play a role in epithelial cell migration and goblet cell exocytosis as a mechanism for shifting bacteria away from the crypt opening. Indeed, microbial dysbiosis and depletion of a number of commensal bacteria is a common finding in UC and CD. Ileal CD is often associated with increases in *Enterobacteriaceae* (such as adherent-invasive strains of *Escherichia coli*) and *Ruminococcus gnavus* populations and a decrease in *Faecalibacterium* and *Roseburia* [189-193, 304]. Interestingly, microbial population remain unaltered with CD disease remission while UC remission induces a microbial population comparable to normal populations [191]. Taken altogether, this study has provided evidence for a sentinel like goblet cell localised to the upper crypt that directs goblet cell expulsion in a NLRP6 dependent manner.

CHAPTER 7 GENERAL DISCUSSION

7.1 DISCUSSION

A contributing factor in the development of intestinal disease is divergent immune responses to commensal bacteria which promote inflammatory pathways that perpetuate chronic inflammation. Since their discovery in 2002, inflammasome complexes have emerged as key regulators of innate immune responses in both health and disease. An accessible framework for investigating the role of inflammasomes in the development of gastrointestinal disease has been provided by murine models of colitis. The increased susceptibility to chemically induced colitis and disease exacerbation in knockout mice with genetic defects in inflammasome related genes highlights the importance of inflammasomes to maintaining mucosal homeostasis. Similar studies investigating the role of inflammasomes in human IBD are currently lacking. This study addresses gaps in the literature by examining the overall expression profiles of inflammasomes in human colitis.

When considering the results contained within this thesis it is worth noting that while UC and CD are grouped under the IBD umbrella they do however, present with contrasting clinical and histological features and divergent disease progression. Crohn's disease is characterised by discontinuous and transmural inflammation and the disease is often complicated by rectal sparing, fistulising disease, bowel strictures, luminal narrowing and abscesses. While in UC, the inflammation is continuous and confined to the mucosa and submucosa layers [11, 297]. Additionally, the mucus layer is thickened in CD and thinned in UC [10].

Chapter 3 details the mRNA expression of inflammasome components in quiescent and active UC and CD. These results demonstrated the upregulation of inflammasome forming receptors, inflammasome components and bacterial sensors which suggests activation of the inflammasome complex. The 131-fold increase in *NLRP6* expression specific to active ileal CD was an exciting and novel finding and is the focus of research presented in Chapter 6. The increased expression of *IL-1β* was consistent with disease activity and the pathological nature of IL-1β. Worth noting is the upregulation of *IL-1β* was concomitant with increased mRNA of other core inflammasome receptors such as *AIM2, NLRP1, NLRP3* and *NLRP6* which makes it impossible to ascertain the contribution of each to the overall production of IL-1β. Indeed, *NLRP3* showed the strongest correlation with *IL-1β* in both active UC and CD. Interestingly, the correlation of *NLRP6* with *IL-1β* and *IL-18* was minimal in active disease. The upregulation of *IL-1β* and downregulation of *PPAR-γ* are well established features of disease activity and confirm the reliability of the mRNA results presented in this study.

Chapter 4, details the localisation of AIM2 in colonic biopsies obtained normal quiescent and active disease. The overall high expression of AIM2 in the epithelial cell and lamina propria immune cells across all IBD phenotypes indicates AIM2 is a key player in orchestrating heightened innate immune responses. The cell specific expression of AIM2 in the intraepithelial lymphocytes is an exciting discovery and suggests a sentinel like function and regulatory role for AIM2. Exploring the role of AIM2 in intraepithelial lymphocytes should be the focus of further research.

Results from Chapter 3 demonstrated an increase in the expression of *NLRP3* in active UC and CD. Chapter 5, details the cellular localisation of NLRP3 and its

spatial relationship to IL-1β. Unlike AIM2, the localisation of NLRP3 in active disease followed a more restricted pattern and was predominantly present in the immune cells of the lamina propria. Interestingly, the epithelial expression of IL-1β observed in the normal colon shifted to cells of the lamina propria during active disease conditions.

NLRP3 is regarded as a global inflammasome sensor, and caspase-1 dependent production of bioactive IL-1β occurs in response to pathogenic, sterile and cellular stress activators. IL-1β is responsible for many of the clinical symptoms such as fever, hypotension, pain and appetite suppression that patients experience during active disease. Locally, IL-1β can induce cytokine production, enhance T cell activation and antigen recognition, and direct neutrophils to the site of injury or infection [50, 204, 205]. When considering the findings of the colocalisation analyses it is first important to understand the histological differences between active UC and active CD. Neutrophil influx into the lamina propria is a common feature of active UC and rarely seen in active CD. As expected, in the normal colon, inflammasome dependent caspase-1 was found to contribute to the production of mature IL-1β. Surprising and novel to this research was the reduced contribution of the NLRP3 inflammasome to IL-1β production in active UC. Given the dominance of a neutrophil lamina propria cell population in active UC, it is possible that neutrophil derive serine proteases contribute more than the NLRP3 inflammasome to the overall IL-1β production (Figure 7-1).

Chapter 6 details research carried on from the disease specific upregulation of *NLRP6* in ileal CD. Previous murine work suggests NLRP6 is multifaceted, and has the ability to regulate goblet cell mucin production and secretion, regulate

epithelial self-renewal and proliferation, protect against chemical induced intestinal injury and tumorigenesis, and negatively regulate inflammasome signaling. With all of this in mind, the research began by examining the localisation of NLRP6 and MUC2 in human IBD. The prominent expression of NLRP6 in ileal CD was localised to the colonic epithelial cell layer, myofibroblasts, neutrophils and monocytic lineage cells of the lamina propria. Previously, NLRP6 expression has been described as being absent from the human colon [295], so to be able to confirm the expression with immunohistochemistry and immunofluorescence confocal microscopy was an important step in the research process. The lack of colocalisation with the major mucin protein, MUC2 and the identification of an apical goblet cell expressing NLRP6 suggested a role for NLRP6 in mucin 2 release. In comparison with murine studies Birchenough et al [289] identified a sentinel goblet cell localised to the crypt opening which undergoes expulsion and compound exocytosis in a NLRP6 dependent manner. The overlap in fluorescence intensity of NLRP6 and the epithelial cell marker, E-cadherin, and the expression in myofibroblasts is intriguing and possibly indicates a role for NLRP6 in epithelial positioning and migration.

The results of Chapter 6 provided evidence for the emerging disparity between human colitis and chemically induced murine colitis. One of the most striking contrasts lies in the activity of the cytokine IL-18. In the murine system the induction of intestinal IL-18 has been shown to be to be NLRP6 dependent [225] and *Nlrp6* deficiency to be related to low levels of IL-18 [129, 186]. This study found no change in the expression of *IL-18* in active UC or active CD despite fluctuations in the expression of *NLRP6.* Taken together, our results suggest *IL-18* expression is not *NLRP6* dependent.

In 2002, Grenier et al [285] provided *in vitro* evidence for the colocalisation of NLRP6 and ASC, which formed punctuated cytoplasmic structures, and the production of IL-1β. However the lack of correlation between *NLRP6* and *IL-1β* or *IL-18* demonstrated in Chapter 3 raises the possibility of NLRP6 functioning independently of inflammasome formation and IL-1β production (Figure 7-2).

In Summary, this study demonstrates two important features of inflammasome activity. Firstly the hierarchical mRNA upregulation of multiple inflammasome complexes in active UC and CD. Secondly, the variations in cellular location of inflammasomes in the colonic mucosa. Taken together, the cell specific functions of individual inflammasome complexes and their cooperation can now be regarded as key events to orchestrating an effective innate immune response. Indeed, activation of the AIM2, NLRP3 and NLRC4 inflammasomes is known to occur upon challenge with *Listeria monocytogenes* and *Candida albicans* [118, 305-308].

In conclusion, these results have provided an insight into the activity of inflammasome in human IBD. Neutrophil-derived serine proteases can now be regarded as a possible source of bioactive IL-1β in active UC, and NLRP6 can now be considered a potential marker for distinguishing ileal CD from colonic CD and terminal ileum involved UC.

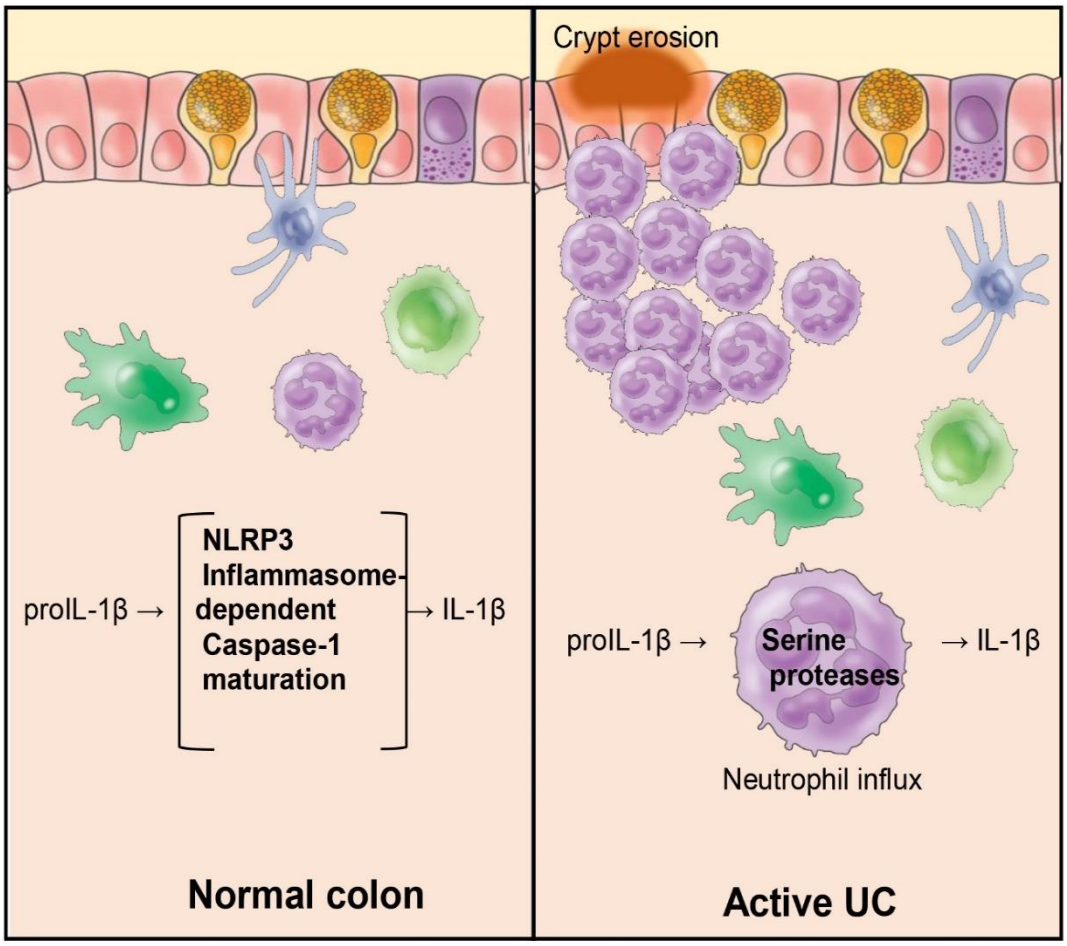


Figure 7-1: Production of IL-1β in the normal colon and active UC

During normal colonic conditions NLRP3 inflammasome dependent caspase-1 is the main contributor to bioactive IL-1β production. In active UC, it is proposed that both inflammasome-dependent caspase-1 and neutrophil-derived serine proteases contribute to the overall production of IL-1β.

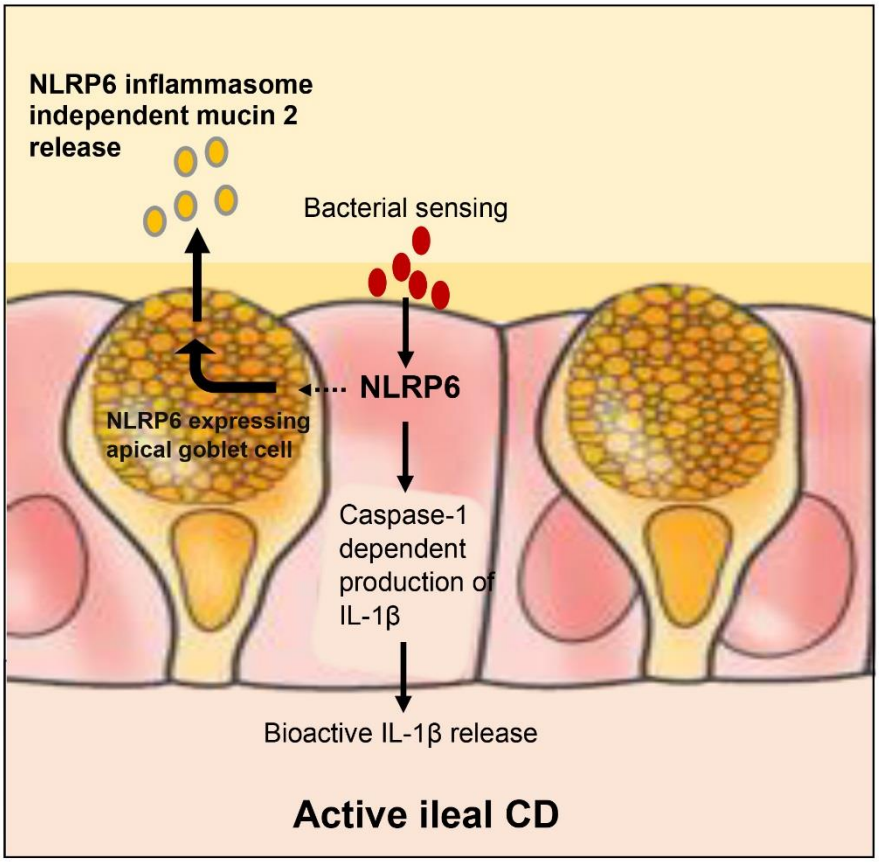


Figure 7-2: Activity of NLRP6 in ileal CD

The increase in NLRP6 mRNA and protein levels in ileal CD represents a possible dual function of NLRP6 at the mucosal surface. It is proposed that NLRP6 is primarily a bacterial sensor of a yet to be determined pathogenic ligand. During high pathogenic loads, NLRP6 influences the release of mucin 2 in an NLRP6-inflamasome-independent manner. The increase in mucus production aids the removal of pathogen from the mucosal surface.

7.2 LIMITATION OF THIS STUDY

This study was reliant on the continued collection of biopsy samples from UC, CD and control patients. Several factors during this process have been identified as affecting the total number of biopsies received, and are detailed below in chronological order.

The task of patient recruitment was given to nursing staff at both the Launceston General Hospital and the Calvary based St Vincent's hospital. Meetings were regularly undertaken to ensure staff were aware of the study, the patient exclusion criteria, informed consent documentation and biopsy collection. However due to the rotational nature and high proportion of casual staff it was difficult to inform all staff. The reluctance to recruit possible participants was most likely a reflection of overworked staff and a lack of information about the study. Interestingly, when staffing was consistent more biopsy samples were collected and a decline was noted when these staff were on annual leave.

During the colonoscopy procedure, patients who provide informed consent were often found to be either, in remission or with disease in its entirety and this led to confusion regarding correct biopsy collection. These biopsies did however contributed to the overall numbers for descriptive statistics but were unsuitable for comparative analyses.

Degraded RNA was the main reason for exclusion of collected biopsy samples and was attributed to delayed preservation of biopsy material. In addition, biopsy samples without informed consent paperwork resulted in biopsies being discarded. Ideally, having the collection site and laboratory in the same facility would allow for better participation of scientific staff/students in the patient recruitment process.

Additionally, budget restrictions prevented whole transcriptome sequencing of IBD patients and therefore failed to provide the depth needed to identify population based SNPs specific to ileal CD or active UC.

7.3 FUTURE DIRECTION

While this study has been important for determining overall activity and cellular distribution of inflammasome components in active disease, the focus of future work should now be directed towards the cell specific action of individual inflammasome complexes. Cell culture provides a cost effective option for studying inflammasome activity and should be used in comparison with human derived colonic tissue wherever possible.

Intraepithelial lymphocytes are positioned within the epithelial cell layer and can perform both sentinel and regulatory functions. Dysregulation of intraepithelial lymphocyte responses can disrupt mucosal barrier integrity and lead to disease pathology [309]. AIM2 is the only inflammasome complex to directly bind its activating ligand, dsDNA. dsDNA is therefore an ideal trigger for examining downstream AIM2 innate responses. Initial studies should include immunofluorescence confocal microscopy with intraepithelial lymphocyte cell markers. Isolation of colonic intraepithelial lymphocytes, challenge with dsDNA and measurements of cytokine levels would potentially identify downstream targets.

The ability of NLRP3 to activate in response to a wide range of stimulants has raised the possibility of NLRP3 blockage as a potential therapeutic option. Indeed, small molecule inhibitors such as MCC950 have shown the ability to block NLRP3 without affecting the activity of the other inflammasomes [284]. Based on this results presented in this study, blocking NLRP3 will not alleviate IL-1β mediated symptoms since IL-1β production is also a result of neutrophil-derived proteases. Future studies investigating NLRP3 in active UC should include immunofluorescence confocal microscopy with neutrophil-derived serine

protease markers to confirm their colocalisation with IL-1β. Isolation of colonic neutrophils, bacterial activation, blocking of individual proteases and measuring the effect on IL-1β and other cytokines would be useful in assessing the contribution of serine protease to downstream inflammatory pathways. Repeating similar investigations in human cell lines may be useful in determining the therapeutic benefit of blocking neutrophil-derived serine proteases in active UC.

The disease specific upregulation of NLRP6 in ileal CD has the greatest potential for ground breaking research. Subsequent studies should examine possible activators of NLRP6 in human cell lines. Unique to future research is the availability of a local ileal CD population with increased NLRP6 expression. Collection of ileal biopsies for explant cultures would provide comparison with human cell lines and may provide insight into downstream pathways or possible treatment options.

APPENDIX 1: CALVARY HEALTH CARE APPROVAL LETTER

Calvary Health Care Tasmania Limited
ABN 29 129 926 790

49 Augusta Road
Lenah Valley TAS 7008
Phone: 03 6278 5333

GPO Box 1523
Hobart TAS 7001

www.calvarycare.org.au

Calvary
Health Care
Tasmania

9 October 2012

Dr Rajaraman D Eri M V Sc., PhD
Private Bag 1320
LAUNCESTON TAS 7250

Dear Dr Eri,

Re: Research Proposal **Pathogenesis of Inflammatory Bowel Disease: Understanding the role of inflammasome**

Our Reference: **03:09:12**

The CHCT Clinical and Research Ethics Committee considered your application to undertake the above project. I am pleased to inform you that after receiving final documentation official approval for this research project has been granted.

Approval is ongoing for the life of the project, subject to satisfactory compliance and reporting. Please be advised that you are obliged to:

- Provide the CHCT Clinical and Research Ethics Committee with an annual report.

In addition, you are obliged to inform the CHCT Clinical and Research Ethics Committee of:

- any change to the protocol, participant information or consent form;
- any adverse events that occur during the process of this trial;
- any changes to the research team;
- study completion; and
- any change in the financial arrangements regarding the study.

Every good wish for the success of your project.

Yours sincerely,

Susie Furphy
Mission Coordinator
Calvary Health Care Tasmania
e: susan.furphy@calvarycare.org.au
m: 0418 490 570

APPENDIX 2: UNIVERSITY OF TASMANIA ETHICS APPROVAL

Office of Research Services
University of Tasmania
Private Bag 1
Hobart Tasmania 7001
Telephone + 61 3 6226 7479
Facsimile + 61 3 6226 7148
Email Human.Ethics@utas.edu.au
www.research.utas.edu.au/human_ethics/

HUMAN RESEARCH ETHICS COMMITTEE (TASMANIA) NETWORK

UTAS

18 May 2012

Dr R Eri
School of Human Life Sciences
University of Tasmania

Sent via email

Dear Dr Eri,

REF NO: H11930
TITLE: Pathogenesis of Inflammatory Bowel disease: Understanding the role of Inflammasome

- *Application Form- NEAF*
- *Patient Participant Information Sheet and Consent Form*
- *Control Participant Information Sheet and Consent Form*
- *Protocol*

The Tasmania Health and Medical Human Research Ethics Committee considered and approved the above documentation on **19 October 2011**.

Please note: this application is approved on the condition that the project does not commence until adequate funding is obtained to enable the project to achieve its aims.

This approval constitutes ethical clearance by the Health and Medical HREC. The decision and authority to commence the associated research may be dependent on factors beyond the remit of the ethics review process. For example, your research may need ethics clearance from other organisations or review by your research governance coordinator or Head of Department. It is your responsibility to find out if the approval of other bodies or authorities are required. It is recommended that the proposed research should not commence until you have satisfied these requirements.

All committees operating under the Human Research Ethics Committee (Tasmania) Network are registered and required to comply with the *National Statement on the Ethical Conduct in Human Research* (NHMRC 2007 updated 2009).

Therefore, the Chief Investigator's responsibility is to ensure that:

(1) The individual researcher's protocol complies with the HREC approved protocol.

(2) Modifications to the protocol do not proceed until **approval** is obtained in writing from the HREC.

(3) Section 5.5.3 of the National Statement states:

Researchers have a significant responsibility in monitoring approved research as they are in the best position to observe any adverse events or unexpected outcomes. They should report such events or outcomes promptly to the relevant institution/s and ethical review body/ies and take prompt steps to deal with any unexpected risks.

The appropriate forms for reporting such events in relation to clinical and non-clinical trials and innovations can be located at the website below. All adverse events must be reported regardless of whether or not the event, in your opinion, is a direct effect of the therapeutic goods being tested. http://www.research.utas.edu.au/human_ethics/medical_forms.htm

(4)All research participants must be provided with the current Patient Information Sheet and Consent Form, unless otherwise approved by the Committee.

(5) The Committee is notified if any investigators are added to, or cease involvement with, the project.

(6) This study has approval for 4 years contingent upon annual review. A *Progress Report* is to be provided on the anniversary date of your approval. Your first report is due **18 October 2012.** You will be sent a courtesy reminder closer to this due date.

(7) A *Final Report* and a copy of the published material, either in full or abstract, must be provided at the end of the project.

Should you have any queries please do not hesitate to contact me on (03) 6226 1956.

Yours sincerely

Adele Kay
Ethics Officer
Health and Medical Human Research Ethics Committee
Human Research Ethics Committee (Tas) Network

APPENDIX 3: PATIENT PARTICIPATION INFORMATION FOR THE IBD STUDY

School of Human Life Sciences, University of Tasmania, Launceston TASMANIA

PATIENT PARTICIPANT INFORMATION TO PARTAKE IN A RESEARCH STUDY

Study Title:
Pathogenesis of inflammatory bowel disease: Understanding the role of inflammasomes
Chief Investigators:
Dr Rajaraman Eri, School of Human Life Sciences, University of Tasmania.
Dr Brent Mitchell, Calvary Health Care, Tasmania.
Dr Scott Fanning, Calvary Health Care, Tasmania.

Introduction
Inflammatory Bowel Diseases (IBD) affect over 60,000 mainly younger Australians and costs close to$3 billion per year to our economy. IBD patients suffer from chronic diarrhoea, intestinal bleeding and abdominal pain affecting the quality of life. The incidence of IBD is on an upward trend in Australia. The cause of IBD is unclear. Recent studies in mouse models of IBD clearly showed a role for a disease causing protein complex known as the Inflammasome. This complex activates two important chemicals-interleukins. This has not been studied in humans.

Aim of the research study
The overall aim of this project is to precisely understand the role of inflammasomes in IBD patients. The specific aim is:
Measure the levels of inflammasome components in biopsies from IBD patients and non-IBD control subjects.

Significance of the planned project
The knowledge gained from this project will aid in understanding how inflammasomes contribute to the development of IBD, which may help in the development of treatments and/or approaches to therapy in patients with IBD.

Reason for invitation to participate in the study
You are being requested to partake in this study because (1) we need to study the disease mechanism from IBD affected colon samples, and (2) your colon biopsy samples will be used as valuable research material for this particular study.

Inclusion criteria
- Age: 15 to 80 years.

Exclusion criteria:

- Age less than 15 years or greater than 80 years.
- Chronic treatment (over 8 weeks) of treatment for IBD.
- Irritable Bowel Syndrome (IBS) or non-IBD associated GI bleeding

Research plan

You will initially be asked to provide information related to your medical history and whether you are currently taking any medications. The information that you provide will be kept strictly confidential. When your IBD clinician performs colonoscopy for your diagnosis, some extra biopsies will be collected specifically for this study as well. These samples will be stored for the duration of the study and will be coded. The collected biopsies will be processed in the research laboratory (Dr Eri at UTAS). Your samples will be tested for the role of protein complexes known as inflammasomes.

What tests will be performed?

The following tests will be performed on the biopsies collected from you:

1. Tests that detect inflammasome elements in the biopsy tissue through molecular biology methods
2. Tests that are done for detecting what type of cells produce these inflammasomes

Which test results will be given to you?

All the results of this study will be analyzed and presented as group data only. These results will be published in scientific medical journal(s) and be discussed at national and international conferences. A summary of results will be available at the end of the study and be provided to you on request. If you would like to know your individual results please contact your IBD clinician.

Confidentiality

All data will be treated in the strictest of confidence. The information that will be collected will only be used for the purpose of this study. Records identifying you will not be made publicly available. If the results of the trial are published in a scientific medical journal, your identity will remain confidential.

Risks and discomforts

Biopsy collection by colonoscopy is a low risk activity that is carried out for usual routine clinical diagnosis of IBD. Your doctor will advise you of the risks and complications of colonoscopy.

You do not need to have a separate colonoscopy for this study, as extra biopsies will be taken during the colonoscopy that you've already been booked for. The additional risks from taking extra biopsies are very minimal, and are no more significant than the routine biopsies that will be taken otherwise.

If you have any further questions, please consult your doctor prior to the procedure.

Cost
Participation in this trial will not result in any costs for you and there is no payment for participation in this study.

Participation
Your participation is voluntary and you may withdraw at any time.

Withdrawal
If you choose to withdraw at any stage, you may request that any, or all, of your data collected for the purpose of this project be destroyed. Any such request will be complied with.

Contact and further information
If you have a question about this study, or would like more information kindly contact
Dr Raj Eri: (03) 6324 5467 or rderi@utas.edu.au, or
Your IBD clinician who recruited you for the study

This study (application number H11930) has been approved by the Human Research Ethics Committee (Tasmania) Network in accordance with the National Health and Medical Research Council's guidelines.

If you have any concerns of an ethical nature or complaints about the manner in which the project is conducted, you may contact the Executive Officer (phone 03 6226 7479) of the Human Research Ethics Committee (Tasmania) Network. The Executive officer can direct participants to the relevant Chair that reviewed the research.

Thank you for your time.

APPENDIX 4: MEDICAL HISTORY AND CONSENT FORM FOR IBD STUDY

School of Human Life Sciences, University of Tasmania, Launceston TASMANIA

MEDICAL HISTORY AND CONSENT FORM
TO PARTAKE IN A RESEARCH STUDY

Study Title:
Pathogenesis of inflammatory bowel disease: Understanding the role of inflammasomes

LABORATORY USE ONLY:	LABORATORY USE ONLY:
PLACE UTAS LABORATORY NUMBER HERE	PLACE LAUNCESTON PATHOLOGY LABORATORY NUMBER HERE

MEDICAL HISTORY:
Prior to providing a sample, please answer the following questions:

1. Gender (tick box): Male ? Female ?
2. Date of Birth (dd/mm/yyyy) ___/___/_____ Current Age (years): _____
3. Are you currently a smoker? Yes ? No ?

   How many/day _____

4. Are you currently taking any prescribed or other medication?

   If yes, please list. Yes ? No ?

1

5. Have you previously been diagnosed with Inflammatory Bowel Disease (also known as IBD- Crohn's disease or Ulcerative Colitis)?

Yes ? No ? If yes, at what age?_____

6. Do you have any other medical problem which you are currently receiving medical attention and/or treatment?

Yes ? No ?

If yes, please provide more details.

APPENDIX 5: SELECTION OF SUITABLE HOUSEKEEPING GENE FOR QRT-PCR NORMALISATION

To determine a suitable housekeeping gene for gene expression normalisation, the mean absolute Ct values for four commonly used reference gene, *EEF2*, *GAPDH*, *HPRT1* and *ACTB* were compared across the 5 disease groups. All of the housekeeping genes demonstrated PCR amplification (Figure A6-1), however EEF2 showed slightly less variability (standard deviation = 0.2328) (Table 7-1) across the disease groups and similar PCR amplification to patient samples and therefore was the gene chosen for normalisation.

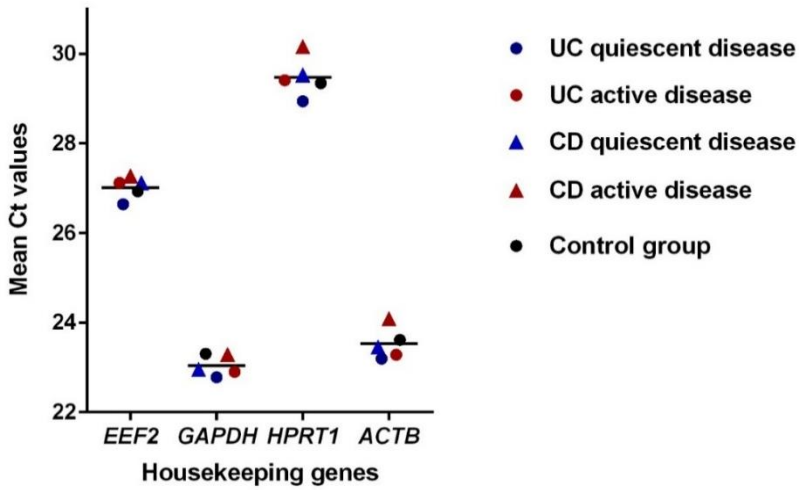


Figure A6-1: Mean absolute Ct values for the four housekeeping genes across the 5 disease categories

Table 7-1: Descriptive statistics for absolute Ct values of the four housekeeping genes

	EEF2	*GAPDH*	*HPRT1*	*ACTB*
Mean Ct	27.01	23.05	29.48	23.53
Standard deviation	0.2328	0.2362	0.4446	0.3532
Standard error of mean	0.1041	0.1056	0.1988	0.1579

APPENDIX 6: PRODUCT INFORMATION SHEET FOR THE AIM2 ANTIBODY (AB93015, ABCAM, CAMBRIDGE, MA)

Product **Datasheet**

Anti-AIM2 antibody ab93015

abcam® discover more

7 Abreviews | 6 References | 3 Images

Overview

Product name	Anti-AIM2 antibody
Description	Rabbit polyclonal to AIM2
Tested applications	WB, IHC-P, ICC
Species reactivity	**Reacts with:** Human
Immunogen	Recombinant fragment, corresponding to amino acids 93-341 of Human AIM2 (NM_004833).
Positive control	EC-109 and HepG2 cell lysates; Human fetal tonsil tissue

Properties

Form	Liquid
Storage instructions	Shipped at 4°C. Upon delivery aliquot and store at -20°C. Avoid repeated freeze / thaw cycles.
Storage buffer	Preservative: 0.02% Sodium Azide Constituents: PBS, pH 7.2
Purity	Immunogen affinity purified
Clonality	Polyclonal
Isotype	IgG

Applications

Our Abpromise guarantee covers the use of **ab93015** in the following tested applications.

The application notes include recommended starting dilutions; optimal dilutions/concentrations should be determined by the end user.

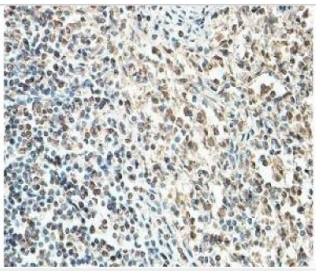
Application	Abreviews	Notes
WB		
IHC-P		
ICC		

Application notes	IHC-P: 1/50 - 1/200. WB: 1/200 - 1/1000. Predicted molecular weight: 39 kDa. Not yet tested in other applications. Optimal dilutions/concentrations should be determined by the end user.

Target

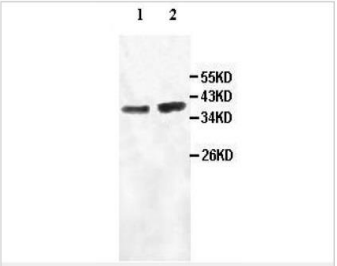
Function	Tumor suppressor which may act by repressing NF-kappa-B transcriptional activity.
Tissue specificity	Expressed in spleen, small intestine, peripheral blood leukocytes, and testis.
Sequence similarities	Belongs to the HIN-200 family. Contains 1 DAPIN domain. Contains 1 HIN-200 domain.
Cellular localization	Nucleus.

Anti-AIM2 antibody images



Immunohistochemistry (Formalin/PFA-fixed paraffin-embedded sections) - AIM2 antibody (ab93015)

Immunohistochemical staining of AIM2 in formalin fixed, paraffin embedded Human fetal tonsil showing nuclear staining with ab93015 at a dilution of 1/100.

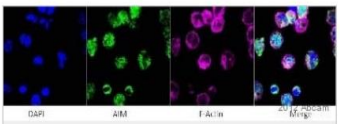


Western blot - AIM2 antibody (ab93015)

All lanes : Anti-AIM2 antibody (ab93015) at 1/500 dilution

Lane 1 : EC-109 cell lysate
Lane 2 : HepG2 cell lysate

Predicted band size : 39 kDa



Immunocytochemistry/ Immunofluorescence - Anti-AIM2 antibody (ab93015)
Image courtesy of Dr Mahesh Shivananjappa by Abreview.

ab93015 staining AIM2 in human monocytes (Apheresis) by Immunocytochemistry/ Immunofluorescence.
Cells were fixed, permeabilized, blocked with 2% BSA for 30 minutes at 25°C and then incubated with ab93015 at a 1/200 dilution for 2 hours at 25°C. The secondary used was an Alexa-Fluor 488 conjugated chicken anti-rabbit IgG (H+L) used at a 1/1000 dilution.

Our Abpromise to you: Quality guaranteed and expert technical support

- Replacement or refund for products not performing as stated on the datasheet
- Valid for 12 months from date of delivery**
- Response to your inquiry within 24 hours
- We provide support in Chinese, English, French, German, Japanese and Spanish
- Extensive multi-media technical resources to help you
- We investigate all quality concerns to ensure our products perform to the highest standards

If the product does not perform as described on this datasheet, we will offer a refund or replacement. For full details of the Abpromise, please visit http://www.abcam.com/abpromise or contact our technical team.

Terms and conditions

APPENDIX 7: PRODUCT INFORMATION SHEET FOR THE NLRP6 ANTIBODY (NBP2-31372, NOVUS BIOLOGICAL, LITTLETON, CO, USA)

Product Datasheet

NALP6 Antibody
NBP2-31372

Unit Size: 0.1 mg

Store at 4C short term. Aliquot and store at -20C long term. Avoid freeze-thaw cycles.

www.novusbio.com support@novusbio.com

Protocols, Publications, Related Products, Reviews, Research Tools and Images at:
www.novusbio.com/NBP2-31372

Updated 3/27/2014 v.20.1

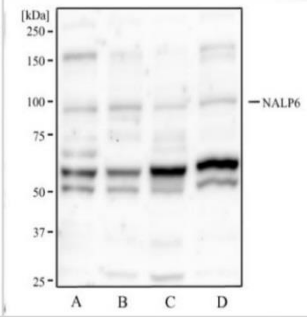
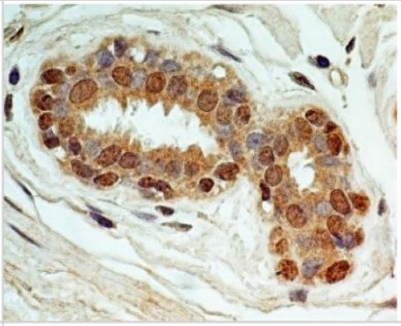
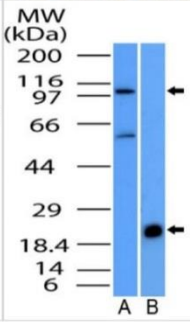
NBP2-31372
NALP6 Antibody

Product Information	
Unit Size	0.1 mg
Concentration	1.0 mg/ml
Storage	Store at 4C short term. Aliquot and store at -20C long term. Avoid freeze-thaw cycles.
Clonality	Polyclonal
Preservative	0.05% Sodium Azide
Purity	Protein A purified
Buffer	PBS

Product Description	
Host	Rabbit
Gene ID	171389
Gene Symbol	GMFG
Species	Human
Species Reactivity	Human. Immunogen sequence similarity with other species: Rat (72%), Mouse (70%)
Immunogen	NALP6

Product Application Details	
Applications	Western Blot, Immunohistochemistry, Immunohistochemistry-Paraffin
Recommended Dilutions	Immunohistochemistry 5 ug/ml , Immunohistochemistry-Paraffin 5 ug/ml , Western Blot 1-2 ug/ml

Images

Western Blot: NALP6 Antibody [NBP2-31372] - Western blot analysis of Hek293 (A), Ntera2 (B), HeLa (C), and K562 (D) cell lysate using NALP6 antibody at 2 ug/ml.	
Immunohistochemistry-Paraffin: NALP6 Antibody [NBP2-31372] - IHC-P analysis of NALP6 protein in a section of human breast normal tissue using NALP6 antibody at a concentration of 5 ug/ml. The breast ductal /acinar epithelium showed a strong NALP6 positivity in the cytoplasm and nuclei of the cells.	
Western Blot: NALP6 Antibody [NBP2-31372] - WB detection of NALP6 protein (NACHT, LRR and PYD domains-containing protein 6) in (A) Jurkat cells lysate and (B) partial recombinant protein using NALP6 antibody at a concentration of 1 ug/ml for lysate and 0.1 ug/ml for the recombinant protein. In Jurkat cells lysate, this antibody detected a major band at ~98.8 kDa which the expected position for NALP6.	

Novus Biologicals USA
8100 Southpark Way, A-8
Littleton, CO 80120
USA
Phone: 303.730.1950
Toll Free: 1.888.506.6887
Fax: 303.730.1966
novus@novusbio.com

Novus Biologicals Canada
461 North Service Road West, Unit B37
Oakville, ON L6M 2V5
Canada
Phone: 905.827.6400
Toll Free: 855.668.8722
Fax: 905.827.6402
canada@novusbio.com

Novus Biologicals Europe
12 Cambridge Science Park
Cambridge, CB4 0FQ
United Kingdom
Phone: +44 (0)1223 426001
Fax: +44 (0)871 971 1635
europe@novusbio.com

General Contact Information
www.novusbio.com
Technical Support: technical@novusbio.com
Orders: orders@novusbio.com
General: novus@novusbio.com

Products Related to NBP2-31372

NB100-56565	Caspase 1 Antibody (14F468)
NBP2-29323	NFkB p105/p50 Inhibitor Peptide Set
NBP1-77080	NALP3 Antibody
NB100-524	NOD2 Antibody (2D9)

Limitations
This product is for research use only and is not approved for use in humans or in clinical diagnosis. Primary Antibodies are guaranteed for 1 year from date of receipt.

For more information on our guarantee, please visit www.novusbio.com/guarantee.

REFERENCES

1. Round, J. L.. Mazmanian, S. K., The gut microbiota shapes intestinal immune responses during health and disease. *Nat Rev Immunol* 2009, 9, (5), 313-23.
2. Cho, J. H., The genetics and immunopathogenesis of inflammatory bowel disease. *Nat Rev Immunol* 2008, 8, (6), 458-66.
3. Alberts, B.,Johnson, A.,Lewis, J.,Martin, R.,Roberts, K.. Walters, P., *Molecular Biology of the cell*. 4th Edition ed.; Garland Science: 2002.
4. Min, Y. W.. Rhee, P. L., The Role of Microbiota on the Gut Immunology. *Clinical therapeutics* 2015, 37, (5), 968-75.
5. Kim, Y. S.. Ho, S. B., Intestinal goblet cells and mucins in health and disease: recent insights and progress. *Current gastroenterology reports* 2010, 12, (5), 319-30.
6. Crohns and Colitis Association of Australia *CCA Annual report 2013*. www.crohnsandcolitis.com.au (viewed 01/02/2018),
7. Loftus, E. V., Jr., Clinical epidemiology of inflammatory bowel disease: Incidence, prevalence, and environmental influences. *Gastroenterology* 2004, 126, (6), 1504-17.
8. Molodecky, N. A.,Soon, I. S.,Rabi, D. M.,Ghali, W. A.,Ferris, M.,Chernoff, G.,Benchimol, E. I.,Panaccione, R.,Ghosh, S.,Barkema, H. W.. Kaplan, G. G., Increasing Incidence and Prevalence of the Inflammatory Bowel Diseases With Time, Based on Systematic Review. *Gastroenterology* 2012, 142, (1), 46-54.e42.

9. Wilson, J.,Hair, C.,Knight, R.,Catto-Smith, A.,Bell, S.,Kamm, M.,Desmond, P.,McNeil, J.. Connell, W., High incidence of inflammatory bowel disease in Australia: a prospective population-based Australian incidence study. *Inflammatory bowel diseases* 2010, 16, (9), 1550-6.

10. Parkes, M.. Jewell, D., Ulcerative colitis and Crohns disease: molecular genetics and clinical implications. *Expert reviews in molecular medicine* 2001, 2001, 1-18.

11. Bickston S.J. Bloomfeld R.S, *Handbook of Inflammatory Bowel Disease*. First Edition ed.; Lippincott Williams and Wilkins, a Wolters Kluwer business: 2010; p 1-175.

12. Sawczenko, A.. Sandhu, B. K., Presenting features of inflammatory bowel disease in Great Britain and Ireland. *Archives of disease in childhood* 2003, 88, (11), 995-1000.

13. Ranson, N.. Eri, R., The Role of Inflammasomes in Intestinal Inflammation. *American Journal of Medical and Biological Research* 2013, 1, (3), 64-76.

14. Magro, F.,Langner, C.,Driessen, A.,Ensari, A.,Geboes, K.,Mantzaris, G. J.,Villanacci, V.,Becheanu, G.,Borralho Nunes, P.,Cathomas, G.,Fries, W.,Jouret-Mourin, A.,Mescoli, C.,de Petris, G.,Rubio, C. A.,Shepherd, N. A.,Vieth, M.,Eliakim, R.,European Society of, P.,European, C. s.. Colitis, O., European consensus on the histopathology of inflammatory bowel disease. *Journal of Crohn's & colitis* 2013, 7, (10), 827-51.

15. D'Haens, G.,Geboes, K.,Peeters, M.,Baert, F.,Ectors, N.. Rutgeerts, P., Patchy cecal inflammation associated with distal ulcerative colitis: a prospective endoscopic study. *The American journal of gastroenterology* 1997, 92, (8), 1275-9.

16. Seldenrijk, C. A.,Morson, B. C.,Meuwissen, S. G.,Schipper, N. W.,Lindeman, J.. Meijer, C. J., Histopathological evaluation of colonic mucosal biopsy specimens in chronic inflammatory bowel disease: diagnostic implications. *Gut* 1991, 32, (12), 1514-20.

17. Tanaka, M.,Saito, H.,Fukuda, S.,Sasaki, Y.,Munakata, A.. Kudo, H., Simple mucosal biopsy criteria differentiating among Crohn disease, ulcerative colitis, and other forms of colitis: measurement of validity. *Scandinavian journal of gastroenterology* 2000, 35, (3), 281-6.

18. D'Haens, G. R.,Sartor, R. B.,Silverberg, M. S.,Petersson, J.. Rutgeerts, P., Future directions in inflammatory bowel disease management. *Journal of Crohn's & colitis* 2014, 8, (8), 726-734.

19. Joo, M.. Odze, R. D., Rectal sparing and skip lesions in ulcerative colitis: a comparative study of endoscopic and histologic findings in patients who underwent proctocolectomy. *The American journal of surgical pathology* 2010, 34, (5), 689-96.

20. Smith, P. D.,Mcdonald, T. T.,Blumberg, R. S.. editors, *Principles of Mucosal Immunology*. Garland Science,Taylor and Francis Group, LLC: 2013.

21. de Lange, K. M.,Moutsianas, L.,Lee, J. C.,Lamb, C. A.,Luo, Y.,Kennedy, N. A.,Jostins, L.,Rice, D. L.,Gutierrez-Achury, J.,Ji, S. G.,Heap, G.,Nimmo, E. R.,Edwards, C.,Henderson, P.,Mowat, C.,Sanderson, J.,Satsangi, J.,Simmons, A.,Wilson, D. C.,Tremelling, M.,Hart, A.,Mathew, C. G.,Newman, W. G.,Parkes, M.,Lees, C. W.,Uhlig, H.,Hawkey, C.,Prescott, N. J.,Ahmad, T.,Mansfield, J. C.,Anderson, C. A.. Barrett, J. C., Genome-wide association study implicates immune activation of multiple integrin genes in inflammatory bowel disease. *Nat Genet* 2017, 49, (2), 256-261.

22. Jostins, L.,Ripke, S.,Weersma, R. K.,Duerr, R. H.,McGovern, D. P.,Hui, K. Y.,Lee, J. C.,Schumm, L. P.,Sharma, Y.,Anderson, C. A.,Essers, J.,Mitrovic, M.,Ning, K.,Cleynen, I.,Theatre, E.,Spain, S. L.,Raychaudhuri, S.,Goyette, P.,Wei, Z.,Abraham, C.,Achkar, J. P.,Ahmad, T.,Amininejad, L.,Ananthakrishnan, A. N.,Andersen, V.,Andrews, J. M.,Baidoo, L.,Balschun, T.,Bampton, P. A.,Bitton, A.,Boucher, G.,Brand, S.,Buning, C.,Cohain, A.,Cichon, S.,D'Amato, M.,De Jong, D.,Devaney, K. L.,Dubinsky, M.,Edwards, C.,Ellinghaus, D.,Ferguson, L. R.,Franchimont, D.,Fransen, K.,Gearry, R.,Georges, M.,Gieger, C.,Glas, J.,Haritunians, T.,Hart, A.,Hawkey, C.,Hedl, M.,Hu, X.,Karlsen, T. H.,Kupcinskas, L.,Kugathasan, S.,Latiano, A.,Laukens, D.,Lawrance, I. C.,Lees, C. W.,Louis, E.,Mahy, G.,Mansfield, J.,Morgan, A. R.,Mowat, C.,Newman, W.,Palmieri, O.,Ponsioen, C. Y.,Potocnik, U.,Prescott, N. J.,Regueiro, M.,Rotter, J. I.,Russell, R. K.,Sanderson, J. D.,Sans, M.,Satsangi, J.,Schreiber, S.,Simms, L. A.,Sventoraityte, J.,Targan, S. R.,Taylor, K. D.,Tremelling, M.,Verspaget, H. W.,De Vos, M.,Wijmenga, C.,Wilson, D. C.,Winkelmann, J.,Xavier, R. J.,Zeissig, S.,Zhang, B.,Zhang, C. K.,Zhao, H.,International, I. B. D. G. C.,Silverberg, M. S.,Annese, V.,Hakonarson, H.,Brant, S. R.,Radford-Smith, G.,Mathew, C. G.,Rioux, J. D.,Schadt, E. E.,Daly, M. J.,Franke, A.,Parkes, M.,Vermeire, S.,Barrett, J. C.. Cho, J. H., Host-microbe interactions have shaped the genetic architecture of inflammatory bowel disease. *Nature* 2012, 491, (7422), 119-24.

23. Cho, J. H.. Weaver, C. T., The genetics of inflammatory bowel disease. *Gastroenterology* 2007, 133, (4), 1327-39.

24. Lees, C. W.,Barrett, J. C.,Parkes, M.. Satsangi, J., New IBD genetics: common pathways with other diseases. *Gut* 2011, 60, (12), 1739-53.

25. Cho, J. H.. Brant, S. R., Recent insights into the genetics of inflammatory bowel disease. *Gastroenterology* 2011, 140, (6), 1704-12.

26. Ng, S. C.,Tang, W.,Leong, R. W.,Chen, M.,Ko, Y.,Studd, C.,Niewiadomski, O.,Bell, S.,Kamm, M. A.,de Silva, H. J.,Kasturiratne, A.,Senanayake, Y. U.,Ooi, C. J.,Ling, K. L.,Ong, D.,Goh, K. L.,Hilmi, I.,Ouyang, Q.,Wang, Y. F.,Hu, P.,Zhu, Z.,Zeng, Z.,Wu, K.,Wang, X.,Xia, B.,Li, J.,Pisespongsa, P.,Manatsathit, S.,Aniwan, S.,Simadibrata, M.,Abdullah, M.,Tsang, S. W.,Wong, T. C.,Hui, A. J.,Chow, C. M.,Yu, H. H.,Li, M. F.,Ng, K. K.,Ching, J.,Wu, J. C.,Chan, F. K.. Sung, J. J., Environmental risk factors in inflammatory bowel disease: a population-based case-control study in Asia-Pacific. *Gut* 2014.

27. Ng, S. C.,Bernstein, C. N.,Vatn, M. H.,Lakatos, P. L.,Loftus, E. V., Jr.,Tysk, C.,O'Morain, C.,Moum, B.,Colombel, J. F.,on behalf of the, E.. Natural History Task Force of the International Organization of Inflammatory Bowel, D., Geographical variability and environmental risk factors in inflammatory bowel disease. *Gut* 2013.

28. Cosnes, J., Smoking, physical activity, nutrition and lifestyle: environmental factors and their impact on IBD. *Digestive diseases* 2010, 28, (3), 411-7.

29. Cosnes, J.,Beaugerie, L.,Carbonnel, F.. Gendre, J. P., Smoking cessation and the course of Crohn's disease: an intervention study. *Gastroenterology* 2001, 120, (5), 1093-9.

30. Johnson, G. J.,Cosnes, J.. Mansfield, J. C., Review article: smoking cessation as primary therapy to modify the course of Crohn's disease. *Alimentary pharmacology & therapeutics* 2005, 21, (8), 921-31.

31. Rosenfeld, G.. Bressler, B., The truth about cigarette smoking and the risk of inflammatory bowel disease. *The American journal of gastroenterology* 2012, 107, (9), 1407-8.

32. Lunney, P. C.. Leong, R. W., Review article: Ulcerative colitis, smoking and nicotine therapy. *Alimentary pharmacology & therapeutics* 2012, 36, (11-12), 997-1008.

33. Naganuma, M.,Iizuka, B.,Torii, A.,Ogihara, T.,Kawamura, Y.,Ichinose, M.,Kojima, Y.,Hibi, T.. Tokyo Gut, C., Appendectomy protects against the development of ulcerative colitis and reduces its recurrence: results of a multicenter case-controlled study in Japan. *The American journal of gastroenterology* 2001, 96, (4), 1123-6.

34. Radford-Smith, G. L.,Edwards, J. E.,Purdie, D. M.,Pandeya, N.,Watson, M.,Martin, N. G.,Green, A.,Newman, B.. Florin, T. H., Protective role of appendicectomy on onset and severity of ulcerative colitis and Crohn's disease. *Gut* 2002, 51, (6), 808-13.

35. Radford-Smith, G. L., What is the importance of appendectomy in the natural history of IBD? *Inflammatory bowel diseases* 2008, 14 Suppl 2, S72-4.

36. Andersson, R. E.,Olaison, G.,Tysk, C.. Ekbom, A., Appendectomy and protection against ulcerative colitis. *The New England journal of medicine* 2001, 344, (11), 808-14.

37. Reif, S.,Lavy, A.,Keter, D.,Broide, E.,Niv, Y.,Halak, A.,Ron, Y.,Eliakim, R.,Odes, S.,Patz, J.,Fich, A.,Villa, Y.,Arber, N.. Gilat, T., Appendectomy is more frequent but not a risk factor in Crohn's disease while being protective in ulcerative colitis: a comparison of surgical procedures in inflammatory

bowel disease. *The American journal of gastroenterology* 2001, 96, (3), 829-32.

38. Sicilia, B.,Lopez Miguel, C.,Arribas, F.,Lopez Zaborras, J.,Sierra, E.. Gomollon, F., Environmental risk factors and Crohn's disease: a population-based, case-control study in Spain. *Digestive and liver disease : official journal of the Italian Society of Gastroenterology and the Italian Association for the Study of the Liver* 2001, 33, (9), 762-7.
39. Garcia Rodriguez, L. A.,Gonzalez-Perez, A.,Johansson, S.. Wallander, M. A., Risk factors for inflammatory bowel disease in the general population. *Alimentary pharmacology & therapeutics* 2005, 22, (4), 309-15.
40. Bernstein, C. N., Why and where to look in the environment with regard to the etiology of inflammatory bowel disease. *Digestive diseases* 2012, 30 Suppl 3, 28-32.
41. Stappenbeck, T. S., The role of autophagy in Paneth cell differentiation and secretion. *Mucosal immunology* 2009, 3, (1), 8-10.
42. Koch, S.. Nusrat, A., Dynamic regulation of epithelial cell fate and barrier function by intercellular junctions. *Annals of the New York Academy of Sciences* 2009, 1165, 220-7.
43. Lechner, J.,Malloth, N.,Seppi, T.,Beer, B.,Jennings, P.. Pfaller, W., IFN-alpha induces barrier destabilization and apoptosis in renal proximal tubular epithelium. *American journal of physiology. Cell physiology* 2008, 294, (1), C153-60.
44. Al-Sadi, R. M.. Ma, T. Y., IL-1beta causes an increase in intestinal epithelial tight junction permeability. *Journal of immunology* 2007, 178, (7), 4641-9.

45. Wyatt, J.,Vogelsang, H.,Hubl, W.,Waldhoer, T.. Lochs, H., Intestinal permeability and the prediction of relapse in Crohn's disease. *Lancet* 1993, 341, (8858), 1437-9.

46. Jorgensen, J.,Ranlov, P. J.,Bjerrum, P. J.,Diemer, H.,Bisgaard, K.. Elsborg, L., Is an increased intestinal permeability a valid predictor of relapse in Crohn disease? *Scandinavian journal of gastroenterology* 2001, 36, (5), 521-7.

47. Miele, E.,Pascarella, F.,Quaglietta, L.,Giannetti, E.,Greco, L.,Troncone, R.. Staiano, A., Altered intestinal permeability is predictive of early relapse in children with steroid-responsive ulcerative colitis. *Alimentary pharmacology & therapeutics* 2007, 25, (8), 933-9.

48. Schroder, K.. Tschopp, J., The inflammasomes. *Cell* 2010, 140, (6), 821-32.

49. Martinon, F.,Mayor, A.. Tschopp, J., The inflammasomes: guardians of the body. *Annual review of immunology* 2009, 27, 229-65.

50. Guarda, G.. So, A., Regulation of inflammasome activity. *Immunology* 2010, 130, (3), 329-36.

51. Fukata, M.. Abreu, M. T., What are toll-like receptors and what role may they have in IBD? *Inflammatory bowel diseases* 2008, 14 Suppl 2, S90-2.

52. Kawai, T.. Akira, S., TLR signaling. *Seminars in immunology* 2007, 19, (1), 24-32.

53. Kawai, T.. Akira, S., Toll-like receptors and their crosstalk with other innate receptors in infection and immunity. *Immunity* 2011, 34, (5), 637-50.

54. Broz, P.. Monack, D. M., Newly described pattern recognition receptors team up against intracellular pathogens. *Nat Rev Immunol* 2013, 13, (8), 551-65.

55. Dinarello, C. A., Interleukin 1 and interleukin 18 as mediators of inflammation and the aging process. *The American journal of clinical nutrition* 2006, 83, (2), 447S-455S.

56. Latz, E.,Xiao, T. S.. Stutz, A., Activation and regulation of the inflammasomes. *Nat Rev Immunol* 2013, 13, (6), 397-411.

57. Lamkanfi, M.,Walle, L. V.. Kanneganti, T. D., Deregulated inflammasome signaling in disease. *Immunological reviews* 2011, 243, (1), 163-73.

58. Lamkanfi, M.. Dixit, V. M., Mechanisms and functions of inflammasomes. *Cell* 2014, 157, (5), 1013-22.

59. Aachoui, Y.,Leaf, I. A.,Hagar, J. A.,Fontana, M. F.,Campos, C. G.,Zak, D. E.,Tan, M. H.,Cotter, P. A.,Vance, R. E.,Aderem, A.. Miao, E. A., Caspase-11 protects against bacteria that escape the vacuole. *Science (New York, N.Y.)* 2013, 339, (6122), 975-8.

60. van de Veerdonk, F. L.,Netea, M. G.,Dinarello, C. A.. Joosten, L. A., Inflammasome activation and IL-1beta and IL-18 processing during infection. *Trends in immunology* 2011, 32, (3), 110-6.

61. Coeshott, C.,Ohnemus, C.,Pilyavskaya, A.,Ross, S.,Wieczorek, M.,Kroona, H.,Leimer, A. H.. Cheronis, J., Converting enzyme-independent release of tumor necrosis factor alpha and IL-1beta from a stimulated human monocytic cell line in the presence of activated neutrophils or purified proteinase 3. *Proceedings of the National Academy of Sciences of the United States of America* 1999, 96, (11), 6261-6.

62. Sugawara, S.,Uehara, A.,Nochi, T.,Yamaguchi, T.,Ueda, H.,Sugiyama, A.,Hanzawa, K.,Kumagai, K.,Okamura, H.. Takada, H., Neutrophil proteinase 3-mediated induction of bioactive IL-18 secretion by human oral epithelial cells. *Journal of immunology* 2001, 167, (11), 6568-75.

63. Guma, M.,Ronacher, L.,Liu-Bryan, R.,Takai, S.,Karin, M.. Corr, M., Caspase 1-independent activation of interleukin-1beta in neutrophil-predominant inflammation. *Arthritis Rheum* 2009, 60, (12), 3642-50.

64. Yazdi, A. S.,Guarda, G.,D'Ombrain, M. C.. Drexler, S. K., Inflammatory caspases in innate immunity and inflammation. *J Innate Immun* 2010, 2, (3), 228-37.

65. Lu, A.,Magupalli, V. G.,Ruan, J.,Yin, Q.,Atianand, M. K.,Vos, M. R.,Schroder, G. F.,Fitzgerald, K. A.,Wu, H.. Egelman, E. H., Unified polymerization mechanism for the assembly of ASC-dependent inflammasomes. *Cell* 2014, 156, (6), 1193-206.

66. de Alba, E., Structure and interdomain dynamics of apoptosis-associated speck-like protein containing a CARD (ASC). *The Journal of biological chemistry* 2009, 284, (47), 32932-41.

67. Bryan, N. B.,Dorfleutner, A.,Kramer, S. J.,Yun, C.,Rojanasakul, Y.. Stehlik, C., Differential splicing of the apoptosis-associated speck like protein containing a caspase recruitment domain (ASC) regulates inflammasomes. *Journal of inflammation* 2010, 7, 23.

68. Rathinam, V. A.,Vanaja, S. K.. Fitzgerald, K. A., Regulation of inflammasome signaling. *Nature immunology* 2012, 13, (4), 333-2.

69. Broz, P.,von Moltke, J.,Jones, J. W.,Vance, R. E.. Monack, D. M., Differential requirement for Caspase-1 autoproteolysis in pathogen-induced cell death and cytokine processing. *Cell host & microbe* 2010, 8, (6), 471-83.

70. Becker, C. E.. O'Neill, L. A., Inflammasomes in inflammatory disorders: the role of TLRs and their interactions with NLRs. *Seminars in immunopathology* 2007, 29, (3), 239-48.

71. Franchi, L.,Munoz-Planillo, R.. Nunez, G., Sensing and reacting to microbes through the inflammasomes. *Nature immunology* 2012, 13, (4), 325-32.

72. Faustin, B.,Lartigue, L.,Bruey, J. M.,Luciano, F.,Sergienko, E.,Bailly-Maitre, B.,Volkmann, N.,Hanein, D.,Rouiller, I.. Reed, J. C., Reconstituted NALP1 inflammasome reveals two-step mechanism of caspase-1 activation. *Molecular cell* 2007, 25, (5), 713-24.

73. D'Osualdo, A.,Weichenberger, C. X.,Wagner, R. N.,Godzik, A.,Wooley, J.. Reed, J. C., CARD8 and NLRP1 undergo autoproteolytic processing through a ZU5-like domain. *PloS one* 2011, 6, (11), e27396.

74. Pathan, N.,Marusawa, H.,Krajewska, M.,Matsuzawa, S.,Kim, H.,Okada, K.,Torii, S.,Kitada, S.,Krajewski, S.,Welsh, K.,Pio, F.,Godzik, A.. Reed, J. C., TUCAN, an antiapoptotic caspase-associated recruitment domain family protein overexpressed in cancer. *The Journal of biological chemistry* 2001, 276, (34), 32220-9.

75. Stilo, R.,Leonardi, A.,Formisano, L.,Di Jeso, B.,Vito, P.. Liguoro, D., TUCAN/CARDINAL and DRAL participate in a common pathway for modulation of NF-kappaB activation. *FEBS letters* 2002, 521, (1-3), 165-9.

76. Tschopp, J.,Martinon, F.. Burns, K., NALPs: a novel protein family involved in inflammation. *Nature reviews. Molecular cell biology* 2003, 4, (2), 95-104.

77. Finger, J. N.,Lich, J. D.,Dare, L. C.,Cook, M. N.,Brown, K. K.,Duraiswami, C.,Bertin, J.. Gough, P. J., Autolytic proteolysis within the function to find domain (FIIND) is required for NLRP1 inflammasome activity. *The Journal of biological chemistry* 2012, 287, (30), 25030-7.

78. Kerur, N.,Veettil, M. V.,Sharma-Walia, N.,Bottero, V.,Sadagopan, S.,Otageri, P.. Chandran, B., IFI16 acts as a nuclear pathogen sensor to induce the inflammasome in response to Kaposi Sarcoma-associated herpesvirus infection. *Cell host & microbe* 2011, 9, (5), 363-75.

79. Schattgen, S. A.. Fitzgerald, K. A., The PYHIN protein family as mediators of host defenses. *Immunological reviews* 2011, 243, (1), 109-18.

80. Cridland, J. A.,Curley, E. Z.,Wykes, M. N.,Schroder, K.,Sweet, M. J.,Roberts, T. L.,Ragan, M. A.,Kassahn, K. S.. Stacey, K. J., The mammalian PYHIN gene family: phylogeny, evolution and expression. *BMC evolutionary biology* 2012, 12, 140.

81. Boyden, E. D.. Dietrich, W. F., Nalp1b controls mouse macrophage susceptibility to anthrax lethal toxin. *Nat Genet* 2006, 38, (2), 240-4.

82. Newman, Z. L.,Printz, M. P.,Liu, S.,Crown, D.,Breen, L.,Miller-Randolph, S.,Flodman, P.,Leppla, S. H.. Moayeri, M., Susceptibility to anthrax lethal toxin-induced rat death is controlled by a single chromosome 10 locus that includes rNlrp1. *PLoS pathogens* 2010, 6, (5), e1000906.

83. Terra, J. K.,Cote, C. K.,France, B.,Jenkins, A. L.,Bozue, J. A.,Welkos, S. L.,LeVine, S. M.. Bradley, K. A., Cutting edge: resistance to Bacillus anthracis

infection mediated by a lethal toxin sensitive allele of Nalp1b/Nlrp1b. *Journal of immunology* 2010, 184, (1), 17-20.

84. Hsu, L. C.,Ali, S. R.,McGillivray, S.,Tseng, P. H.,Mariathasan, S.,Humke, E. W.,Eckmann, L.,Powell, J. J.,Nizet, V.,Dixit, V. M.. Karin, M., A NOD2-NALP1 complex mediates caspase-1-dependent IL-1beta secretion in response to Bacillus anthracis infection and muramyl dipeptide. *Proceedings of the National Academy of Sciences of the United States of America* 2008, 105, (22), 7803-8.

85. Maeda, S.,Hsu, L. C.,Liu, H.,Bankston, L. A.,Iimura, M.,Kagnoff, M. F.,Eckmann, L.. Karin, M., Nod2 mutation in Crohn's disease potentiates NF-kappaB activity and IL-1beta processing. *Science (New York, N.Y.)* 2005, 307, (5710), 734-8.

86. Anand, P. K.,Malireddi, R. K.. Kanneganti, T. D., Role of the nlrp3 inflammasome in microbial infection. *Frontiers in microbiology* 2011, 2, 12.

87. Munoz-Planillo, R.,Kuffa, P.,Martinez-Colon, G.,Smith, B. L.,Rajendiran, T. M.. Nunez, G., K(+) efflux is the common trigger of NLRP3 inflammasome activation by bacterial toxins and particulate matter. *Immunity* 2013, 38, (6), 1142-53.

88. Zhou, R.,Tardivel, A.,Thorens, B.,Choi, I.. Tschopp, J., Thioredoxin-interacting protein links oxidative stress to inflammasome activation. *Nature immunology* 2010, 11, (2), 136-40.

89. Hornung, V.,Bauernfeind, F.,Halle, A.,Samstad, E. O.,Kono, H.,Rock, K. L.,Fitzgerald, K. A.. Latz, E., Silica crystals and aluminum salts activate the

NALP3 inflammasome through phagosomal destabilization. *Nature immunology* 2008, 9, (8), 847-56.

90. Shimada, K.,Crother, T. R.,Karlin, J.,Dagvadorj, J.,Chiba, N.,Chen, S.,Ramanujan, V. K.,Wolf, A. J.,Vergnes, L.,Ojcius, D. M.,Rentsendorj, A.,Vargas, M.,Guerrero, C.,Wang, Y.,Fitzgerald, K. A.,Underhill, D. M.,Town, T.. Arditi, M., Oxidized mitochondrial DNA activates the NLRP3 inflammasome during apoptosis. *Immunity* 2012, 36, (3), 401-14.

91. Nakahira, K.,Haspel, J. A.,Rathinam, V. A.,Lee, S. J.,Dolinay, T.,Lam, H. C.,Englert, J. A.,Rabinovitch, M.,Cernadas, M.,Kim, H. P.,Fitzgerald, K. A.,Ryter, S. W.. Choi, A. M., Autophagy proteins regulate innate immune responses by inhibiting the release of mitochondrial DNA mediated by the NALP3 inflammasome. *Nature immunology* 2011, 12, (3), 222-30.

92. Iyer, S. S.,He, Q.,Janczy, J. R.,Elliott, E. I.,Zhong, Z.,Olivier, A. K.,Sadler, J. J.,Knepper-Adrian, V.,Han, R.,Qiao, L.,Eisenbarth, S. C.,Nauseef, W. M.,Cassel, S. L.. Sutterwala, F. S., Mitochondrial cardiolipin is required for Nlrp3 inflammasome activation. *Immunity* 2013, 39, (2), 311-23.

93. Misawa, T.,Takahama, M.,Kozaki, T.,Lee, H.,Zou, J.,Saitoh, T.. Akira, S., Microtubule-driven spatial arrangement of mitochondria promotes activation of the NLRP3 inflammasome. *Nature immunology* 2013, 14, (5), 454-60.

94. Subramanian, N.,Natarajan, K.,Clatworthy, M. R.,Wang, Z.. Germain, R. N., The adaptor MAVS promotes NLRP3 mitochondrial localization and inflammasome activation. *Cell* 2013, 153, (2), 348-61.

95. Bauernfeind, F. G.,Horvath, G.,Stutz, A.,Alnemri, E. S.,MacDonald, K.,Speert, D.,Fernandes-Alnemri, T.,Wu, J.,Monks, B. G.,Fitzgerald, K. A.,Hornung, V..

Latz, E., Cutting edge: NF-kappaB activating pattern recognition and cytokine receptors license NLRP3 inflammasome activation by regulating NLRP3 expression. *Journal of immunology* 2009, 183, (2), 787-91.

96. Franchi, L.,Eigenbrod, T.. Nunez, G., Cutting edge: TNF-alpha mediates sensitization to ATP and silica via the NLRP3 inflammasome in the absence of microbial stimulation. *Journal of immunology* 2009, 183, (2), 792-6.

97. Gombault, A.,Baron, L.. Couillin, I., ATP release and purinergic signaling in NLRP3 inflammasome activation. *Frontiers in immunology* 2012, 3, 414.

98. Shenoy, A. R.,Wellington, D. A.,Kumar, P.,Kassa, H.,Booth, C. J.,Cresswell, P.. MacMicking, J. D., GBP5 promotes NLRP3 inflammasome assembly and immunity in mammals. *Science (New York, N.Y.)* 2012, 336, (6080), 481-5.

99. Martinon, F.,Petrilli, V.,Mayor, A.,Tardivel, A.. Tschopp, J., Gout-associated uric acid crystals activate the NALP3 inflammasome. *Nature* 2006, 440, (7081), 237-41.

100. Rajamaki, K.,Lappalainen, J.,Oorni, K.,Valimaki, E.,Matikainen, S.,Kovanen, P. T.. Eklund, K. K., Cholesterol crystals activate the NLRP3 inflammasome in human macrophages: a novel link between cholesterol metabolism and inflammation. *PloS one* 2010, 5, (7), e11765.

101. Halle, A.,Hornung, V.,Petzold, G. C.,Stewart, C. R.,Monks, B. G.,Reinheckel, T.,Fitzgerald, K. A.,Latz, E.,Moore, K. J.. Golenbock, D. T., The NALP3 inflammasome is involved in the innate immune response to amyloid-beta. *Nature immunology* 2008, 9, (8), 857-65.

102. Duncan, J. A.,Gao, X.,Huang, M. T.,O'Connor, B. P.,Thomas, C. E.,Willingham, S. B.,Bergstralh, D. T.,Jarvis, G. A.,Sparling, P. F.. Ting, J. P., Neisseria gonorrhoeae activates the proteinase cathepsin B to mediate the signaling activities of the NLRP3 and ASC-containing inflammasome. *Journal of immunology* 2009, 182, (10), 6460-9.

103. Dostert, C.,Guarda, G.,Romero, J. F.,Menu, P.,Gross, O.,Tardivel, A.,Suva, M. L.,Stehle, J. C.,Kopf, M.,Stamenkovic, I.,Corradin, G.. Tschopp, J., Malarial hemozoin is a Nalp3 inflammasome activating danger signal. *PloS one* 2009, 4, (8), e6510.

104. Sena, L. A.. Chandel, N. S., Physiological roles of mitochondrial reactive oxygen species. *Molecular cell* 2012, 48, (2), 158-67.

105. Zhou, R.,Yazdi, A. S.,Menu, P.. Tschopp, J., A role for mitochondria in NLRP3 inflammasome activation. *Nature* 2011, 469, (7329), 221-5.

106. Petrilli, V.,Papin, S.,Dostert, C.,Mayor, A.,Martinon, F.. Tschopp, J., Activation of the NALP3 inflammasome is triggered by low intracellular potassium concentration. *Cell death and differentiation* 2007, 14, (9), 1583-9.

107. Dostert, C.,Petrilli, V.,Van Bruggen, R.,Steele, C.,Mossman, B. T.. Tschopp, J., Innate immune activation through Nalp3 inflammasome sensing of asbestos and silica. *Science (New York, N.Y.)* 2008, 320, (5876), 674-7.

108. Kawai, T.,Takahashi, K.,Sato, S.,Coban, C.,Kumar, H.,Kato, H.,Ishii, K. J.,Takeuchi, O.. Akira, S., IPS-1, an adaptor triggering RIG-I- and Mda5-mediated type I interferon induction. *Nature immunology* 2005, 6, (10), 981-8.

109. Li, X. D.,Chiu, Y. H.,Ismail, A. S.,Behrendt, C. L.,Wight-Carter, M.,Hooper, L. V.. Chen, Z. J., Mitochondrial antiviral signaling protein (MAVS) monitors commensal bacteria and induces an immune response that prevents experimental colitis. *Proceedings of the National Academy of Sciences of the United States of America* 2011, 108, (42), 17390-5.

110. Lamkanfi, M.,Mueller, J. L.,Vitari, A. C.,Misaghi, S.,Fedorova, A.,Deshayes, K.,Lee, W. P.,Hoffman, H. M.. Dixit, V. M., Glyburide inhibits the Cryopyrin/Nalp3 inflammasome. *The Journal of cell biology* 2009, 187, (1), 61-70.

111. Bauernfeind, F.,Ablasser, A.,Bartok, E.,Kim, S.,Schmid-Burgk, J.,Cavlar, T.. Hornung, V., Inflammasomes: current understanding and open questions. *Cellular and molecular life sciences : CMLS* 2011, 68, (5), 765-83.

112. Franchi, L.,Kanneganti, T. D.,Dubyak, G. R.. Nunez, G., Differential requirement of P2X7 receptor and intracellular K+ for caspase-1 activation induced by intracellular and extracellular bacteria. *The Journal of biological chemistry* 2007, 282, (26), 18810-8.

113. Harder, J.,Franchi, L.,Munoz-Planillo, R.,Park, J. H.,Reimer, T.. Nunez, G., Activation of the Nlrp3 inflammasome by Streptococcus pyogenes requires streptolysin O and NF-kappa B activation but proceeds independently of TLR signaling and P2X7 receptor. *Journal of immunology* 2009, 183, (9), 5823-9.

114. Franchi, L.,Amer, A.,Body-Malapel, M.,Kanneganti, T. D.,Ozoren, N.,Jagirdar, R.,Inohara, N.,Vandenabeele, P.,Bertin, J.,Coyle, A.,Grant, E. P.. Nunez, G., Cytosolic flagellin requires Ipaf for activation of caspase-1 and

interleukin 1beta in salmonella-infected macrophages. *Nature immunology* 2006, 7, (6), 576-82.

115. Miao, E. A.,Alpuche-Aranda, C. M.,Dors, M.,Clark, A. E.,Bader, M. W.,Miller, S. I.. Aderem, A., Cytoplasmic flagellin activates caspase-1 and secretion of interleukin 1beta via Ipaf. *Nature immunology* 2006, 7, (6), 569-75.

116. Miao, E. A.,Mao, D. P.,Yudkovsky, N.,Bonneau, R.,Lorang, C. G.,Warren, S. E.,Leaf, I. A.. Aderem, A., Innate immune detection of the type III secretion apparatus through the NLRC4 inflammasome. *Proceedings of the National Academy of Sciences of the United States of America* 2010, 107, (7), 3076-80.

117. Yang, J.,Zhao, Y.,Shi, J.. Shao, F., Human NAIP and mouse NAIP1 recognize bacterial type III secretion needle protein for inflammasome activation. *Proceedings of the National Academy of Sciences of the United States of America* 2013, 110, (35), 14408-13.

118. Wu, J.,Fernandes-Alnemri, T.. Alnemri, E. S., Involvement of the AIM2, NLRC4, and NLRP3 inflammasomes in caspase-1 activation by Listeria monocytogenes. *Journal of clinical immunology* 2010, 30, (5), 693-702.

119. Zhao, Y.,Yang, J.,Shi, J.,Gong, Y. N.,Lu, Q.,Xu, H.,Liu, L.. Shao, F., The NLRC4 inflammasome receptors for bacterial flagellin and type III secretion apparatus. *Nature* 2011, 477, (7366), 596-600.

120. Fernandes-Alnemri, T.,Yu, J. W.,Datta, P.,Wu, J.. Alnemri, E. S., AIM2 activates the inflammasome and cell death in response to cytoplasmic DNA. *Nature* 2009, 458, (7237), 509-13.

121. Hornung, V.,Ablasser, A.,Charrel-Dennis, M.,Bauernfeind, F.,Horvath, G.,Caffrey, D. R.,Latz, E.. Fitzgerald, K. A., AIM2 recognizes cytosolic dsDNA

and forms a caspase-1-activating inflammasome with ASC. *Nature* 2009, 458, (7237), 514-8.

122. Chu, X.,Chen, W.,Li, N.,Hu, X. Z.,Du, C. T.,Yu, S. X.,Zhou, M.,Zhang, X. J.,Jiang, G. M.,Han, W. Y.,Deng, X. M.. Yang, Y. J., Cytosolic double-stranded DNA induces nonnecroptotic programmed cell death in trophoblasts via IFI16. *The Journal of infectious diseases* 2014, 210, (9), 1476-86.

123. Jin, T.,Perry, A.,Jiang, J.,Smith, P.,Curry, J. A.,Unterholzner, L.,Jiang, Z.,Horvath, G.,Rathinam, V. A.,Johnstone, R. W.,Hornung, V.,Latz, E.,Bowie, A. G.,Fitzgerald, K. A.. Xiao, T. S., Structures of the HIN domain:DNA complexes reveal ligand binding and activation mechanisms of the AIM2 inflammasome and IFI16 receptor. *Immunity* 2012, 36, (4), 561-71.

124. Muruve, D. A.,Petrilli, V.,Zaiss, A. K.,White, L. R.,Clark, S. A.,Ross, P. J.,Parks, R. J.. Tschopp, J., The inflammasome recognizes cytosolic microbial and host DNA and triggers an innate immune response. *Nature* 2008, 452, (7183), 103-7.

125. Fernandes-Alnemri, T.,Yu, J. W.,Juliana, C.,Solorzano, L.,Kang, S.,Wu, J.,Datta, P.,McCormick, M.,Huang, L.,McDermott, E.,Eisenlohr, L.,Landel, C. P.. Alnemri, E. S., The AIM2 inflammasome is critical for innate immunity to Francisella tularensis. *Nature immunology* 2010, 11, (5), 385-93.

126. Jones, J. W.,Kayagaki, N.,Broz, P.,Henry, T.,Newton, K.,O'Rourke, K.,Chan, S.,Dong, J.,Qu, Y.,Roose-Girma, M.,Dixit, V. M.. Monack, D. M., Absent in melanoma 2 is required for innate immune recognition of Francisella tularensis. *Proceedings of the National Academy of Sciences of the United States of America* 2010, 107, (21), 9771-6.

127. Rathinam, V. A.,Jiang, Z.,Waggoner, S. N.,Sharma, S.,Cole, L. E.,Waggoner, L.,Vanaja, S. K.,Monks, B. G.,Ganesan, S.,Latz, E.,Hornung, V.,Vogel, S. N.,Szomolanyi-Tsuda, E.. Fitzgerald, K. A., The AIM2 inflammasome is essential for host defense against cytosolic bacteria and DNA viruses. *Nature immunology* 2010, 11, (5), 395-402.

128. Chen, G. Y., Role of Nlrp6 and Nlrp12 in the maintenance of intestinal homeostasis. *European journal of immunology* 2014, 44, (2), 321-7.

129. Chen, G. Y.,Liu, M.,Wang, F.,Bertin, J.. Nunez, G., A functional role for Nlrp6 in intestinal inflammation and tumorigenesis. *Journal of immunology* 2011, 186, (12), 7187-94.

130. Anand, P. K.,Malireddi, R. K.,Lukens, J. R.,Vogel, P.,Bertin, J.,Lamkanfi, M.. Kanneganti, T. D., NLRP6 negatively regulates innate immunity and host defence against bacterial pathogens. *Nature* 2012, 488, (7411), 389-93.

131. Khare, S.,Dorfleutner, A.,Bryan, N. B.,Yun, C.,Radian, A. D.,de Almeida, L.,Rojanasakul, Y.. Stehlik, C., An NLRP7-containing inflammasome mediates recognition of microbial lipopeptides in human macrophages. *Immunity* 2012, 36, (3), 464-76.

132. Martinon, F.. Tschopp, J., Inflammatory caspases and inflammasomes: master switches of inflammation. *Cell death and differentiation* 2007, 14, (1), 10-22.

133. Mayor, A.,Martinon, F.,De Smedt, T.,Petrilli, V.. Tschopp, J., A crucial function of SGT1 and HSP90 in inflammasome activity links mammalian and plant innate immune responses. *Nature immunology* 2007, 8, (5), 497-503.

134. Py, B. F.,Kim, M. S.,Vakifahmetoglu-Norberg, H.. Yuan, J., Deubiquitination of NLRP3 by BRCC3 critically regulates inflammasome activity. *Molecular cell* 2013, 49, (2), 331-8.

135. Qu, Y.,Misaghi, S.,Izrael-Tomasevic, A.,Newton, K.,Gilmour, L. L.,Lamkanfi, M.,Louie, S.,Kayagaki, N.,Liu, J.,Komuves, L.,Cupp, J. E.,Arnott, D.,Monack, D.. Dixit, V. M., Phosphorylation of NLRC4 is critical for inflammasome activation. *Nature* 2012, 490, (7421), 539-42.

136. Bruey, J. M.,Bruey-Sedano, N.,Luciano, F.,Zhai, D.,Balpai, R.,Xu, C.,Kress, C. L.,Bailly-Maitre, B.,Li, X.,Osterman, A.,Matsuzawa, S.,Terskikh, A. V.,Faustin, B.. Reed, J. C., Bcl-2 and Bcl-XL regulate proinflammatory caspase-1 activation by interaction with NALP1. *Cell* 2007, 129, (1), 45-56.

137. Faustin, B.,Chen, Y.,Zhai, D.,Le Negrate, G.,Lartigue, L.,Satterthwait, A.. Reed, J. C., Mechanism of Bcl-2 and Bcl-X(L) inhibition of NLRP1 inflammasome: loop domain-dependent suppression of ATP binding and oligomerization. *Proceedings of the National Academy of Sciences of the United States of America* 2009, 106, (10), 3935-40.

138. Arlehamn, C. S.,Petrilli, V.,Gross, O.,Tschopp, J.. Evans, T. J., The role of potassium in inflammasome activation by bacteria. *The Journal of biological chemistry* 2010, 285, (14), 10508-18.

139. da Cunha, J. P.,Galante, P. A.. de Souza, S. J., Different evolutionary strategies for the origin of caspase-1 inhibitors. *Journal of molecular evolution* 2008, 66, (6), 591-7.

140. Stehlik, C.,Krajewska, M.,Welsh, K.,Krajewski, S.,Godzik, A.. Reed, J. C., The PAAD/PYRIN-only protein POP1/ASC2 is a modulator of ASC-mediated

nuclear-factor-kappa B and pro-caspase-1 regulation. *The Biochemical journal* 2003, 373, (Pt 1), 101-13.

141. Bedoya, F.,Sandler, L. L.. Harton, J. A., Pyrin-only protein 2 modulates NF-kappaB and disrupts ASC:CLR interactions. *Journal of immunology* 2007, 178, (6), 3837-45.

142. Stehlik, C.. Dorfleutner, A., COPs and POPs: modulators of inflammasome activity. *Journal of immunology* 2007, 179, (12), 7993-8.

143. Lamkanfi, M.,Denecker, G.,Kalai, M.,D'Hondt, K.,Meeus, A.,Declercq, W.,Saelens, X.. Vandenabeele, P., INCA, a novel human caspase recruitment domain protein that inhibits interleukin-1beta generation. *The Journal of biological chemistry* 2004, 279, (50), 51729-38.

144. Guarda, G.,Braun, M.,Staehli, F.,Tardivel, A.,Mattmann, C.,Forster, I.,Farlik, M.,Decker, T.,Du Pasquier, R. A.,Romero, P.. Tschopp, J., Type I interferon inhibits interleukin-1 production and inflammasome activation. *Immunity* 2011, 34, (2), 213-23.

145. Guarda, G.,Dostert, C.,Staehli, F.,Cabalzar, K.,Castillo, R.,Tardivel, A.,Schneider, P.. Tschopp, J., T cells dampen innate immune responses through inhibition of NLRP1 and NLRP3 inflammasomes. *Nature* 2009, 460, (7252), 269-73.

146. Allen, I. C.,Wilson, J. E.,Schneider, M.,Lich, J. D.,Roberts, R. A.,Arthur, J. C.,Woodford, R. M.,Davis, B. K.,Uronis, J. M.,Herfarth, H. H.,Jobin, C.,Rogers, A. B.. Ting, J. P., NLRP12 suppresses colon inflammation and tumorigenesis through the negative regulation of noncanonical NF-kappaB signaling. *Immunity* 2012, 36, (5), 742-54.

147. Imamura, R.,Wang, Y.,Kinoshita, T.,Suzuki, M.,Noda, T.,Sagara, J.,Taniguchi, S.,Okamoto, H.. Suda, T., Anti-inflammatory activity of PYNOD and its mechanism in humans and mice. *Journal of immunology* 2010, 184, (10), 5874-84.

148. Wang, Y.,Hasegawa, M.,Imamura, R.,Kinoshita, T.,Kondo, C.,Konaka, K.. Suda, T., PYNOD, a novel Apaf-1/CED4-like protein is an inhibitor of ASC and caspase-1. *International immunology* 2004, 16, (6), 777-86.

149. Kinoshita, T.,Wang, Y.,Hasegawa, M.,Imamura, R.. Suda, T., PYPAF3, a PYRIN-containing APAF-1-like protein, is a feedback regulator of caspase-1-dependent interleukin-1beta secretion. *The Journal of biological chemistry* 2005, 280, (23), 21720-5.

150. Messaed, C.,Akoury, E.,Djuric, U.,Zeng, J.,Saleh, M.,Gilbert, L.,Seoud, M.,Qureshi, S.. Slim, R., NLRP7, a nucleotide oligomerization domain-like receptor protein, is required for normal cytokine secretion and co-localizes with Golgi and the microtubule-organizing center. *The Journal of biological chemistry* 2011, 286, (50), 43313-23.

151. Ozaki, E.,Campbell, M.. Doyle, S. L., Targeting the NLRP3 inflammasome in chronic inflammatory diseases: current perspectives. *Journal of inflammation research* 2015, 8, 15-27.

152. Paramel, G. V.,Sirsjo, A.. Fransen, K., Role of genetic alterations in the NLRP3 and CARD8 genes in health and disease. *Mediators of inflammation* 2015, 2015, 846782.

153. Villani, A. C.,Lemire, M.,Fortin, G.,Louis, E.,Silverberg, M. S.,Collette, C.,Baba, N.,Libioulle, C.,Belaiche, J.,Bitton, A.,Gaudet, D.,Cohen, A.,Langelier, D.,Fortin, P. R.,Wither, J. E.,Sarfati, M.,Rutgeerts, P.,Rioux, J. D.,Vermeire,

S.,Hudson, T. J.. Franchimont, D., Common variants in the NLRP3 region contribute to Crohn's disease susceptibility. *Nat Genet* 2009, 41, (1), 71-6.

154. Lewis, G. J.,Massey, D. C.,Zhang, H.,Bredin, F.,Tremelling, M.,Lee, J. C.,Berzuini, C.. Parkes, M., Genetic association between NLRP3 variants and Crohn's disease does not replicate in a large UK panel. *Inflammatory bowel diseases* 2011, 17, (6), 1387-91.

155. Yang, S. K.,Kim, H.,Hong, M.,Lim, J.,Choi, E.,Ye, B. D.,Park, S. K.. Song, K., Association of CARD8 with inflammatory bowel disease in Koreans. *Journal of human genetics* 2011, 56, (3), 217-23.

156. Schoultz, I.,Verma, D.,Halfvarsson, J.,Torkvist, L.,Fredrikson, M.,Sjoqvist, U.,Lordal, M.,Tysk, C.,Lerm, M.,Soderkvist, P.. Soderholm, J. D., Combined polymorphisms in genes encoding the inflammasome components NALP3 and CARD8 confer susceptibility to Crohn's disease in Swedish men. *The American journal of gastroenterology* 2009, 104, (5), 1180-8.

157. Ungerback, J.,Belenki, D.,Jawad ul-Hassan, A.,Fredrikson, M.,Fransen, K.,Elander, N.,Verma, D.. Soderkvist, P., Genetic variation and alterations of genes involved in NFkappaB/TNFAIP3- and NLRP3-inflammasome signaling affect susceptibility and outcome of colorectal cancer. *Carcinogenesis* 2012, 33, (11), 2126-34.

158. Dwivedi, M.,Laddha, N. C.,Mansuri, M. S.,Marfatia, Y. S.. Begum, R., Association of NLRP1 genetic variants and mRNA overexpression with generalized vitiligo and disease activity in a Gujarat population. *The British journal of dermatology* 2013, 169, (5), 1114-25.

159. Pontillo, A.,Catamo, E.,Arosio, B.,Mari, D.. Crovella, S., NALP1/NLRP1 genetic variants are associated with Alzheimer disease. *Alzheimer disease and associated disorders* 2012, 26, (3), 277-81.

160. Zurawek, M.,Fichna, M.,Januszkiewicz-Lewandowska, D.,Gryczynska, M.,Fichna, P.. Nowak, J., A coding variant in NLRP1 is associated with autoimmune Addison's disease. *Human immunology* 2010, 71, (5), 530-4.

161. Magitta, N. F.,Boe Wolff, A. S.,Johansson, S.,Skinningsrud, B.,Lie, B. A.,Myhr, K. M.,Undlien, D. E.,Joner, G.,Njolstad, P. R.,Kvien, T. K.,Forre, O.,Knappskog, P. M.. Husebye, E. S., A coding polymorphism in NALP1 confers risk for autoimmune Addison's disease and type 1 diabetes. *Genes and immunity* 2009, 10, (2), 120-4.

162. Jin, Y.,Birlea, S. A.,Fain, P. R.. Spritz, R. A., Genetic variations in NALP1 are associated with generalized vitiligo in a Romanian population. *The Journal of investigative dermatology* 2007, 127, (11), 2558-62.

163. Geldhoff, M.,Mook-Kanamori, B. B.,Brouwer, M. C.,Valls Seron, M.,Baas, F.,van der Ende, A.. van de Beek, D., Genetic variation in inflammasome genes is associated with outcome in bacterial meningitis. *Immunogenetics* 2013, 65, (1), 9-16.

164. McGovern, D. P.,Butler, H.,Ahmad, T.,Paolucci, M.,van Heel, D. A.,Negoro, K.,Hysi, P.,Ragoussis, J.,Travis, S. P.,Cardon, L. R.. Jewell, D. P., TUCAN (CARD8) genetic variants and inflammatory bowel disease. *Gastroenterology* 2006, 131, (4), 1190-6.

165. Roberts, R. L.,Topless, R. K.,Phipps-Green, A. J.,Gearry, R. B.,Barclay, M. L.. Merriman, T. R., Evidence of interaction of CARD8 rs2043211 with NALP3 rs35829419 in Crohn's disease. *Genes and immunity* 2010, 11, (4), 351-6.

166. Thompson, S. R.. Humphries, S. E., Interleukin-18 genetics and inflammatory disease susceptibility. *Genes and immunity* 2007, 8, (2), 91-9.

167. Siegmund, B., Interleukin-18 in intestinal inflammation: friend and foe? *Immunity* 2010, 32, (3), 300-2.

168. Nolan, K. F.,Greaves, D. R.. Waldmann, H., The human interleukin 18 gene IL18 maps to 11q22.2-q22.3, closely linked to the DRD2 gene locus and distinct from mapped IDDM loci. *Genomics* 1998, 51, (1), 161-3.

169. Tamura, K.,Fukuda, Y.,Sashio, H.,Takeda, N.,Bamba, H.,Kosaka, T.,Fukui, S.,Sawada, K.,Tamura, K.,Satomi, M.,Yamada, T.,Yamamura, T.,Yamamoto, Y.,Furuyama, J.,Okamura, H.. Shimoyama, T., IL18 polymorphism is associated with an increased risk of Crohn's disease. *Journal of gastroenterology* 2002, 37 Suppl 14, 111-6.

170. Takagawa, T.,Tamura, K.,Takeda, N.,Tomita, T.,Ohda, Y.,Fukunaga, K.,Hida, N.,Ohnishi, K.,Hori, K.,Kosaka, T.,Fukuda, Y.,Ikeuchi, H.,Yamamura, T.,Miwa, H.. Matsumoto, T., Association between IL-18 gene promoter polymorphisms and inflammatory bowel disease in a Japanese population. *Inflammatory bowel diseases* 2005, 11, (12), 1038-43.

171. Haas, S. L.,Andreas Koch, W.,Schreiber, S.,Reinhard, I.,Koyama, N.,Singer, M. V.. Bocker, U., -137 (G/C) IL-18 promoter polymorphism in patients with inflammatory bowel disease. *Scandinavian journal of gastroenterology* 2005, 40, (12), 1438-43.

172. Ben Aleya, W.,Sfar, I.,Habibi, I.,Mouelhi, L.,Aouadi, H.,Makhlouf, M.,Ayed-Jendoubi, S.,Najjar, T.,Ben Abdallah, T.,Ayed, K.. Gorgi, Y., Interleukin-18

gene polymorphisms in tunisian patients with inflammatory bowel disease. *Digestion* 2011, 83, (4), 269-74.

173. Zhernakova, A.,Festen, E. M.,Franke, L.,Trynka, G.,van Diemen, C. C.,Monsuur, A. J.,Bevova, M.,Nijmeijer, R. M.,van 't Slot, R.,Heijmans, R.,Boezen, H. M.,van Heel, D. A.,van Bodegraven, A. A.,Stokkers, P. C.,Wijmenga, C.,Crusius, J. B.. Weersma, R. K., Genetic analysis of innate immunity in Crohn's disease and ulcerative colitis identifies two susceptibility loci harboring CARD9 and IL18RAP. *American journal of human genetics* 2008, 82, (5), 1202-10.

174. Wang, Y.,Tong, J.,Chang, B.,Wang, B. F.,Zhang, D.. Wang, B. Y., Genetic polymorphisms in the IL-18 gene and ulcerative colitis risk: a meta-analysis. *DNA and cell biology* 2014, 33, (7), 438-47.

175. Macaluso, F.,Nothnagel, M.,Parwez, Q.,Petrasch-Parwez, E.,Bechara, F. G.,Epplen, J. T.. Hoffjan, S., Polymorphisms in NACHT-LRR (NLR) genes in atopic dermatitis. *Experimental dermatology* 2007, 16, (8), 692-8.

176. Roy, N.,Mahadevan, M. S.,McLean, M.,Shutler, G.,Yaraghi, Z.,Farahani, R.,Baird, S.,Besner-Johnston, A.,Lefebvre, C.,Kang, X.. et al., The gene for neuronal apoptosis inhibitory protein is partially deleted in individuals with spinal muscular atrophy. *Cell* 1995, 80, (1), 167-78.

177. Romberg, N.,Al Moussawi, K.,Nelson-Williams, C.,Stiegler, A. L.,Loring, E.,Choi, M.,Overton, J.,Meffre, E.,Khokha, M. K.,Huttner, A. J.,West, B.,Podoltsev, N. A.,Boggon, T. J.,Kazmierczak, B. I.. Lifton, R. P., Mutation of NLRC4 causes a syndrome of enterocolitis and autoinflammation. *Nat Genet* 2014, 46, (10), 1135-9.

178. Canna, S. W.,de Jesus, A. A.,Gouni, S.,Brooks, S. R.,Marrero, B.,Liu, Y.,DiMattia, M. A.,Zaal, K. J.,Sanchez, G. A.,Kim, H.,Chapelle, D.,Plass, N.,Huang, Y.,Villarino, A. V.,Biancotto, A.,Fleisher, T. A.,Duncan, J. A.,O'Shea, J. J.,Benseler, S.,Grom, A.,Deng, Z.,Laxer, R. M.. Goldbach-Mansky, R., An activating NLRC4 inflammasome mutation causes autoinflammation with recurrent macrophage activation syndrome. *Nat Genet* 2014, 46, (10), 1140-6.

179. Dupaul-Chicoine, J.,Yeretssian, G.,Doiron, K.,Bergstrom, K. S.,McIntire, C. R.,LeBlanc, P. M.,Meunier, C.,Turbide, C.,Gros, P.,Beauchemin, N.,Vallance, B. A.. Saleh, M., Control of intestinal homeostasis, colitis, and colitis-associated colorectal cancer by the inflammatory caspases. *Immunity* 2010, 32, (3), 367-78.

180. Li, P.,Allen, H.,Banerjee, S.,Franklin, S.,Herzog, L.,Johnston, C.,McDowell, J.,Paskind, M.,Rodman, L.,Salfeld, J.. et al., Mice deficient in IL-1 beta-converting enzyme are defective in production of mature IL-1 beta and resistant to endotoxic shock. *Cell* 1995, 80, (3), 401-11.

181. Bauer, C.,Duewell, P.,Mayer, C.,Lehr, H. A.,Fitzgerald, K. A.,Dauer, M.,Tschopp, J.,Endres, S.,Latz, E.. Schnurr, M., Colitis induced in mice with dextran sulfate sodium (DSS) is mediated by the NLRP3 inflammasome. *Gut* 2010, 59, (9), 1192-9.

182. Kuida, K.,Lippke, J. A.,Ku, G.,Harding, M. W.,Livingston, D. J.,Su, M. S.. Flavell, R. A., Altered cytokine export and apoptosis in mice deficient in interleukin-1 beta converting enzyme. *Science (New York, N.Y.)* 1995, 267, (5206), 2000-3.

183. Zaki, M. H.,Boyd, K. L.,Vogel, P.,Kastan, M. B.,Lamkanfi, M.. Kanneganti, T. D., The NLRP3 inflammasome protects against loss of epithelial integrity and mortality during experimental colitis. *Immunity* 2010, 32, (3), 379-91.

184. Hirota, S. A.,Ng, J.,Lueng, A.,Khajah, M.,Parhar, K.,Li, Y.,Lam, V.,Potentier, M. S.,Ng, K.,Bawa, M.,McCafferty, D. M.,Rioux, K. P.,Ghosh, S.,Xavier, R. J.,Colgan, S. P.,Tschopp, J.,Muruve, D.,MacDonald, J. A.. Beck, P. L., NLRP3 inflammasome plays a key role in the regulation of intestinal homeostasis. *Inflammatory bowel diseases* 2011, 17, (6), 1359-72.

185. Allen, I. C.,TeKippe, E. M.,Woodford, R. M.,Uronis, J. M.,Holl, E. K.,Rogers, A. B.,Herfarth, H. H.,Jobin, C.. Ting, J. P., The NLRP3 inflammasome functions as a negative regulator of tumorigenesis during colitis-associated cancer. *The Journal of experimental medicine* 2010, 207, (5), 1045-56.

186. Elinav, E.,Strowig, T.,Kau, A. L.,Henao-Mejia, J.,Thaiss, C. A.,Booth, C. J.,Peaper, D. R.,Bertin, J.,Eisenbarth, S. C.,Gordon, J. I.. Flavell, R. A., NLRP6 inflammasome regulates colonic microbial ecology and risk for colitis. *Cell* 2011, 145, (5), 745-57.

187. Wlodarska, M.,Thaiss, C. A.,Nowarski, R.,Henao-Mejia, J.,Zhang, J. P.,Brown, E. M.,Frankel, G.,Levy, M.,Katz, M. N.,Philbrick, W. M.,Elinav, E.,Finlay, B. B.. Flavell, R. A., NLRP6 inflammasome orchestrates the colonic host-microbial interface by regulating goblet cell mucus secretion. *Cell* 2014, 156, (5), 1045-59.

188. Mamantopoulos, M.,Ronchi, F.,Van Hauwermeiren, F.,Vieira-Silva, S.,Yilmaz, B.,Martens, L.,Saeys, Y.,Drexler, S. K.,Yazdi, A. S.,Raes, J.,Lamkanfi, M.,McCoy, K. D.. Wullaert, A., Nlrp6- and ASC-Dependent Inflammasomes

Do Not Shape the Commensal Gut Microbiota Composition. *Immunity* 2017.

189. Swidsinski, A.,Loening-Baucke, V.,Vaneechoutte, M.. Doerffel, Y., Active Crohn's disease and ulcerative colitis can be specifically diagnosed and monitored based on the biostructure of the fecal flora. *Inflammatory bowel diseases* 2008, 14, (2), 147-61.

190. Frank, D. N.,St Amand, A. L.,Feldman, R. A.,Boedeker, E. C.,Harpaz, N.. Pace, N. R., Molecular-phylogenetic characterization of microbial community imbalances in human inflammatory bowel diseases. *Proceedings of the National Academy of Sciences of the United States of America* 2007, 104, (34), 13780-5.

191. Willing, B. P.,Dicksved, J.,Halfvarson, J.,Andersson, A. F.,Lucio, M.,Zheng, Z.,Jarnerot, G.,Tysk, C.,Jansson, J. K.. Engstrand, L., A pyrosequencing study in twins shows that gastrointestinal microbial profiles vary with inflammatory bowel disease phenotypes. *Gastroenterology* 2010, 139, (6), 1844-1854 e1.

192. Martin, H. M.,Campbell, B. J.,Hart, C. A.,Mpofu, C.,Nayar, M.,Singh, R.,Englyst, H.,Williams, H. F.. Rhodes, J. M., Enhanced Escherichia coli adherence and invasion in Crohn's disease and colon cancer. *Gastroenterology* 2004, 127, (1), 80-93.

193. Neut, C.,Bulois, P.,Desreumaux, P.,Membre, J. M.,Lederman, E.,Gambiez, L.,Cortot, A.,Quandalle, P.,van Kruiningen, H.. Colombel, J. F., Changes in the bacterial flora of the neoterminal ileum after ileocolonic resection for Crohn's disease. *The American journal of gastroenterology* 2002, 97, (4), 939-46.

194. Elinav, E.,Henao-Mejia, J.. Flavell, R. A., Integrative inflammasome activity in the regulation of intestinal mucosal immune responses. *Mucosal immunology* 2013, 6, (1), 4-13.

195. Elinav, E.,Strowig, T.,Henao-Mejia, J.. Flavell, R. A., Regulation of the antimicrobial response by NLR proteins. *Immunity* 2011, 34, (5), 665-79.

196. Chamaillard, M.,Hashimoto, M.,Horie, Y.,Masumoto, J.,Qiu, S.,Saab, L.,Ogura, Y.,Kawasaki, A.,Fukase, K.,Kusumoto, S.,Valvano, M. A.,Foster, S. J.,Mak, T. W.,Nunez, G.. Inohara, N., An essential role for NOD1 in host recognition of bacterial peptidoglycan containing diaminopimelic acid. *Nature immunology* 2003, 4, (7), 702-7.

197. Girardin, S. E.,Boneca, I. G.,Carneiro, L. A.,Antignac, A.,Jehanno, M.,Viala, J.,Tedin, K.,Taha, M. K.,Labigne, A.,Zahringer, U.,Coyle, A. J.,DiStefano, P. S.,Bertin, J.,Sansonetti, P. J.. Philpott, D. J., Nod1 detects a unique muropeptide from gram-negative bacterial peptidoglycan. *Science (New York, N.Y.)* 2003, 300, (5625), 1584-7.

198. Girardin, S. E.,Travassos, L. H.,Herve, M.,Blanot, D.,Boneca, I. G.,Philpott, D. J.,Sansonetti, P. J.. Mengin-Lecreulx, D., Peptidoglycan molecular requirements allowing detection by Nod1 and Nod2. *The Journal of biological chemistry* 2003, 278, (43), 41702-8.

199. Coll, R. C.. O'Neill, L. A. J., New Insights into the Regulation of Signalling by Toll-Like Receptors and Nod-Like Receptors. *Journal of Innate Immunity* 2010, 2, (5), 406-421.

200. Sabbah, A.,Chang, T. H.,Harnack, R.,Frohlich, V.,Tominaga, K.,Dube, P. H.,Xiang, Y.. Bose, S., Activation of innate immune antiviral responses by Nod2. *Nature immunology* 2009, 10, (10), 1073-80.

201. Inohara, N.,Ogura, Y.,Fontalba, A.,Gutierrez, O.,Pons, F.,Crespo, J.,Fukase, K.,Inamura, S.,Kusumoto, S.,Hashimoto, M.,Foster, S. J.,Moran, A. P.,Fernandez-Luna, J. L.. Nunez, G., Host recognition of bacterial muramyl dipeptide mediated through NOD2. Implications for Crohn's disease. *The Journal of biological chemistry* 2003, 278, (8), 5509-12.

202. Girardin, S. E.,Boneca, I. G.,Viala, J.,Chamaillard, M.,Labigne, A.,Thomas, G.,Philpott, D. J.. Sansonetti, P. J., Nod2 is a general sensor of peptidoglycan through muramyl dipeptide (MDP) detection. *The Journal of biological chemistry* 2003, 278, (11), 8869-72.

203. Lamkanfi, M.. Dixit, V. M., Inflammasomes: guardians of cytosolic sanctity. *Immunological reviews* 2009, 227, (1), 95-105.

204. Kanneganti, T. D., Central roles of NLRs and inflammasomes in viral infection. *Nat Rev Immunol* 2010, 10, (10), 688-98.

205. Sahoo, M.,Ceballos-Olvera, I.,del Barrio, L.. Re, F., Role of the inflammasome, IL-1beta, and IL-18 in bacterial infections. *TheScientificWorldJournal* 2011, 11, 2037-50.

206. Monteleone, G.,Trapasso, F.,Parrello, T.,Biancone, L.,Stella, A.,Iuliano, R.,Luzza, F.,Fusco, A.. Pallone, F., Bioactive IL-18 expression is up-regulated in Crohn's disease. *Journal of immunology* 1999, 163, (1), 143-7.

207. Kuppala, M. B.,Syed, S. B.,Bandaru, S.,Varre, S.,Akka, J.. Mundulru, H. P., Immunotherapeutic approach for better management of cancer - role of IL-18. *Asian Pacific journal of cancer prevention : APJCP* 2012, 13, (11), 5353-61.

208. Stuyt, R. J.,Netea, M. G.,Geijtenbeek, T. B.,Kullberg, B. J.,Dinarello, C. A.. van der Meer, J. W., Selective regulation of intercellular adhesion molecule-1 expression by interleukin-18 and interleukin-12 on human monocytes. *Immunology* 2003, 110, (3), 329-34.

209. Su, C. G.,Wen, X.,Bailey, S. T.,Jiang, W.,Rangwala, S. M.,Keilbaugh, S. A.,Flanigan, A.,Murthy, S.,Lazar, M. A.. Wu, G. D., A novel therapy for colitis utilizing PPAR-gamma ligands to inhibit the epithelial inflammatory response. *The Journal of clinical investigation* 1999, 104, (4), 383-9.

210. Tyagi, S.,Gupta, P.,Saini, A. S.,Kaushal, C.. Sharma, S., The peroxisome proliferator-activated receptor: A family of nuclear receptors role in various diseases. *J Adv Pharm Technol Res* 2011, 2, (4), 236-40.

211. Horlein, A. J.,Naar, A. M.,Heinzel, T.,Torchia, J.,Gloss, B.,Kurokawa, R.,Ryan, A.,Kamei, Y.,Soderstrom, M.,Glass, C. K.. et al., Ligand-independent repression by the thyroid hormone receptor mediated by a nuclear receptor co-repressor. *Nature* 1995, 377, (6548), 397-404.

212. Kempster, S. L.,Belteki, G.,Forhead, A. J.,Fowden, A. L.,Catalano, R. D.,Lam, B. Y.,McFarlane, I.,Charnock-Jones, D. S.. Smith, G. C., Developmental control of the Nlrp6 inflammasome and a substrate, IL-18, in mammalian intestine. *American journal of physiology. Gastrointestinal and liver physiology* 2011, 300, (2), 253-263.

213. Strober, W.,Murray, P. J.,Kitani, A.. Watanabe, T., Signalling pathways and molecular interactions of NOD1 and NOD2. *Nat Rev Immunol* 2006, 6, (1), 9-20.

214. McAlindon, M. E.,Hawkey, C. J.. Mahida, Y. R., Expression of interleukin 1 beta and interleukin 1 beta converting enzyme by intestinal macrophages in health and inflammatory bowel disease. *Gut* 1998, 42, (2), 214-9.

215. Reinecker, H. C.,Steffen, M.,Witthoeft, T.,Pflueger, I.,Schreiber, S.,MacDermott, R. P.. Raedler, A., Enhanced secretion of tumour necrosis factor-alpha, IL-6, and IL-1 beta by isolated lamina propria mononuclear cells from patients with ulcerative colitis and Crohn's disease. *Clinical and experimental immunology* 1993, 94, (1), 174-81.

216. Chang, Y. Y.. Ouyang, Q., [Expression and significance of mucosal beta-defensin-2, TNFalpha and IL-1beta in ulcerative colitis]. *Zhonghua nei ke za zhi [Chinese journal of internal medicine]* 2008, 47, (1), 11-4.

217. Ligumsky, M.,Simon, P. L.,Karmeli, F.. Rachmilewitz, D., Role of interleukin 1 in inflammatory bowel disease--enhanced production during active disease. *Gut* 1990, 31, (6), 686-9.

218. Masumoto, J.,Kobayashi, H.,Nakamura, T.,Kaneko, Y.,Ota, H.,Hasegawa, M.,Kobayashi, Y.,Suzuki, T.,Matsuda, K.,Sano, K.,Katsuyama, T.. Inohara, N., Regulation of the ASC expression in response to LPS stimulation is related to IL-8 secretion in the human intestinal mucosa. *Biochemical and biophysical research communications* 2006, 346, (3), 968-73.

219. Hasegawa, M.,Imamura, R.,Motani, K.,Nishiuchi, T.,Matsumoto, N.,Kinoshita, T.. Suda, T., Mechanism and repertoire of ASC-mediated gene expression. *Journal of immunology* 2009, 182, (12), 7655-62.

220. Christophi, G. P.,Rong, R.,Holtzapple, P. G.,Massa, P. T.. Landas, S. K., Immune markers and differential signaling networks in ulcerative colitis

and Crohn's disease. *Inflammatory bowel diseases* 2012, 18, (12), 2342-56.

221. Alipour, M.,Zaidi, D.,Valcheva, R.,Jovel, J.,Martinez, I.,Sergi, C.,Walter, J.,Mason, A. L.,Wong, G. K.,Dieleman, L. A.,Carroll, M. W.,Huynh, H. Q.. Wine, E., Mucosal Barrier Depletion and Loss of Bacterial Diversity are Primary Abnormalities in Paediatric Ulcerative Colitis. *Journal of Crohn's & colitis* 2015.

222. Kanai, T.,Watanabe, M.,Okazawa, A.,Nakamaru, K.,Okamoto, M.,Naganuma, M.,Ishii, H.,Ikeda, M.,Kurimoto, M.. Hibi, T., Interleukin 18 is a potent proliferative factor for intestinal mucosal lymphocytes in Crohn's disease. *Gastroenterology* 2000, 119, (6), 1514-23.

223. Pizarro, T. T.,Michie, M. H.,Bentz, M.,Woraratanadharm, J.,Smith, M. F., Jr.,Foley, E.,Moskaluk, C. A.,Bickston, S. J.. Cominelli, F., IL-18, a novel immunoregulatory cytokine, is up-regulated in Crohn's disease: expression and localization in intestinal mucosal cells. *Journal of immunology* 1999, 162, (11), 6829-35.

224. Zhang, B.,Lu, Y.,Hong, T.,Pei, F.,Li, F.. Wang, X., [Expression of interleukin 18 in intestinal mucosa of patients with inflammatory bowel disease and its implications]. *Beijing da xue xue bao. Yi xue ban = Journal of Peking University. Health sciences* 2003, 35, (2), 150-3.

225. Levy, M.,Thaiss, C. A.,Zeevi, D.,Dohnalova, L.,Zilberman-Schapira, G.,Mahdi, J. A.,David, E.,Savidor, A.,Korem, T.,Herzig, Y.,Pevsner-Fischer, M.,Shapiro, H.,Christ, A.,Harmelin, A.,Halpern, Z.,Latz, E.,Flavell, R. A.,Amit, I.,Segal, E.. Elinav, E., Microbiota-Modulated Metabolites Shape the Intestinal

Microenvironment by Regulating NLRP6 Inflammasome Signaling. *Cell* 2015, 163, (6), 1428-43.

226. Normand, S.,Delanoye-Crespin, A.,Bressenot, A.,Huot, L.,Grandjean, T.,Peyrin-Biroulet, L.,Lemoine, Y.,Hot, D.. Chamaillard, M., Nod-like receptor pyrin domain-containing protein 6 (NLRP6) controls epithelial self-renewal and colorectal carcinogenesis upon injury. *Proceedings of the National Academy of Sciences of the United States of America* 2011, 108, (23), 9601-6.

227. Dou, X.,Xiao, J.,Jin, Z.. Zheng, P., Peroxisome proliferator-activated receptor-gamma is downregulated in ulcerative colitis and is involved in experimental colitis-associated neoplasia. *Oncology letters* 2015, 10, (3), 1259-1266.

228. Dubuquoy, L.,Jansson, E. A.,Deeb, S.,Rakotobe, S.,Karoui, M.,Colombel, J. F.,Auwerx, J.,Pettersson, S.. Desreumaux, P., Impaired expression of peroxisome proliferator-activated receptor gamma in ulcerative colitis. *Gastroenterology* 2003, 124, (5), 1265-76.

229. Stronati, L.,Negroni, A.,Merola, P.,Pannone, V.,Borrelli, O.,Cirulli, M.,Annese, V.. Cucchiara, S., Mucosal NOD2 expression and NF-kappaB activation in pediatric Crohn's disease. *Inflammatory bowel diseases* 2008, 14, (3), 295-302.

230. Rosenstiel, P.,Fantini, M.,Brautigam, K.,Kuhbacher, T.,Waetzig, G. H.,Seegert, D.. Schreiber, S., TNF-alpha and IFN-gamma regulate the expression of the NOD2 (CARD15) gene in human intestinal epithelial cells. *Gastroenterology* 2003, 124, (4), 1001-9.

231. Hisamatsu, T.,Suzuki, M.,Reinecker, H. C.,Nadeau, W. J.,McCormick, B. A.. Podolsky, D. K., CARD15/NOD2 functions as an antibacterial factor in human intestinal epithelial cells. *Gastroenterology* 2003, 124, (4), 993-1000.

232. Gutierrez, O.,Pipaon, C.,Inohara, N.,Fontalba, A.,Ogura, Y.,Prosper, F.,Nunez, G.. Fernandez-Luna, J. L., Induction of Nod2 in myelomonocytic and intestinal epithelial cells via nuclear factor-kappa B activation. *The Journal of biological chemistry* 2002, 277, (44), 41701-5.

233. Hisamatsu, T.,Suzuki, M.. Podolsky, D. K., Interferon-gamma augments CARD4/NOD1 gene and protein expression through interferon regulatory factor-1 in intestinal epithelial cells. *The Journal of biological chemistry* 2003, 278, (35), 32962-8.

234. Kim, J. G.,Lee, S. J.. Kagnoff, M. F., Nod1 is an essential signal transducer in intestinal epithelial cells infected with bacteria that avoid recognition by toll-like receptors. *Infection and immunity* 2004, 72, (3), 1487-95.

235. Hedl, M.. Abraham, C., The NLRP1 and NLRP3 Inflammasome is Essential for Distinct Outcomes of Decreased Cytokines but Enhanced Bacterial Killing Upon Chronic Nod2 Stimulation. *American journal of physiology. Gastrointestinal and liver physiology* 2013.

236. Ogura, Y.,Lala, S.,Xin, W.,Smith, E.,Dowds, T. A.,Chen, F. F.,Zimmermann, E.,Tretiakova, M.,Cho, J. H.,Hart, J.,Greenson, J. K.,Keshav, S.. Nunez, G., Expression of NOD2 in Paneth cells: a possible link to Crohn's ileitis. *Gut* 2003, 52, (11), 1591-7.

237. Lala, S.,Ogura, Y.,Osborne, C.,Hor, S. Y.,Bromfield, A.,Davies, S.,Ogunbiyi, O.,Nunez, G.. Keshav, S., Crohn's disease and the NOD2 gene: a role for paneth cells. *Gastroenterology* 2003, 125, (1), 47-57.

238. Wehkamp, J.,Harder, J.,Weichenthal, M.,Schwab, M.,Schaffeler, E.,Schlee, M.,Herrlinger, K. R.,Stallmach, A.,Noack, F.,Fritz, P.,Schroder, J. M.,Bevins, C. L.,Fellermann, K.. Stange, E. F., NOD2 (CARD15) mutations in Crohn's disease are associated with diminished mucosal alpha-defensin expression. *Gut* 2004, 53, (11), 1658-64.

239. Stacey, K. J.,Sweet, M. J.. Hume, D. A., Macrophages ingest and are activated by bacterial DNA. *Journal of immunology* 1996, 157, (5), 2116-22.

240. Stetson, D. B.. Medzhitov, R., Recognition of cytosolic DNA activates an IRF3-dependent innate immune response. *Immunity* 2006, 24, (1), 93-103.

241. Hemmi, H.,Takeuchi, O.,Kawai, T.,Kaisho, T.,Sato, S.,Sanjo, H.,Matsumoto, M.,Hoshino, K.,Wagner, H.,Takeda, K.. Akira, S., A Toll-like receptor recognizes bacterial DNA. *Nature* 2000, 408, (6813), 740-5.

242. Marques, R.. Boneca, I. G., Expression and functional importance of innate immune receptors by intestinal epithelial cells. *Cellular and molecular life sciences : CMLS* 2011, 68, (22), 3661-73.

243. Stacey, K. J.,Ross, I. L.. Hume, D. A., Electroporation and DNA-dependent cell death in murine macrophages. *Immunology and cell biology* 1993, 71 (Pt 2), 75-85.

244. Roberts, T. L.,Idris, A.,Dunn, J. A.,Kelly, G. M.,Burnton, C. M.,Hodgson, S.,Hardy, L. L.,Garceau, V.,Sweet, M. J.,Ross, I. L.,Hume, D. A.. Stacey, K. J.,

HIN-200 proteins regulate caspase activation in response to foreign cytoplasmic DNA. *Science (New York, N.Y.)* 2009, 323, (5917), 1057-60.

245. Man, S. M.,Zhu, Q.,Zhu, L.,Liu, Z.,Karki, R.,Malik, A.,Sharma, D.,Li, L.,Malireddi, R. K.,Gurung, P.,Neale, G.,Olsen, S. R.,Carter, R. A.,McGoldrick, D. J.,Wu, G.,Finkelstein, D.,Vogel, P.,Gilbertson, R. J.. Kanneganti, T. D., Critical Role for the DNA Sensor AIM2 in Stem Cell Proliferation and Cancer. *Cell* 2015, 162, (1), 45-58.

246. Schulmann, K.,Brasch, F. E.,Kunstmann, E.,Engel, C.,Pagenstecher, C.,Vogelsang, H.,Kruger, S.,Vogel, T.,Knaebel, H. P.,Ruschoff, J.,Hahn, S. A.,Knebel-Doeberitz, M. V.,Moeslein, G.,Meltzer, S. J.,Schackert, H. K.,Tympner, C.,Mangold, E.,Schmiegel, W.. German, H. C., HNPCC-associated small bowel cancer: clinical and molecular characteristics. *Gastroenterology* 2005, 128, (3), 590-9.

247. Dihlmann, S.,Tao, S.,Echterdiek, F.,Herpel, E.,Jansen, L.,Chang-Claude, J.,Brenner, H.,Hoffmeister, M.. Kloor, M., Lack of Absent in Melanoma 2 (AIM2) expression in tumor cells is closely associated with poor survival in colorectal cancer patients. *International journal of cancer. Journal international du cancer* 2014, 135, (10), 2387-96.

248. Ponomareva, L.,Liu, H.,Duan, X.,Dickerson, E.,Shen, H.,Panchanathan, R.. Choubey, D., AIM2, an IFN-inducible cytosolic DNA sensor, in the development of benign prostate hyperplasia and prostate cancer. *Mol Cancer Res* 2013, 11, (10), 1193-202.

249. Choubey, D.,Walter, S.,Geng, Y.. Xin, H., Cytoplasmic localization of the interferon-inducible protein that is encoded by the AIM2 (absent in

melanoma) gene from the 200-gene family. *FEBS letters* 2000, 474, (1), 38-42.

250. Lugrin, J.. Martinon, F., The AIM2 inflammasome: Sensor of pathogens and cellular perturbations. *Immunological reviews* 2018, 281, (1), 99-114.

251. Choubey, D.. Panchanathan, R., Comment on "Deficient NLRP3 and AIM2 Inflammasome Function in Autoimmune NZB Mice". *Journal of immunology* 2015, 195, (10), 4551-2.

252. Sester, D. P.,Sagulenko, V.,Thygesen, S. J.,Cridland, J. A.,Loi, Y. S.,Cridland, S. O.,Masters, S. L.,Genske, U.,Hornung, V.,Andoniou, C. E.,Sweet, M. J.,Degli-Esposti, M. A.,Schroder, K.. Stacey, K. J., Deficient NLRP3 and AIM2 Inflammasome Function in Autoimmune NZB Mice. *Journal of immunology* 2015, 195, (3), 1233-41.

253. Sester, D. P.. Stacey, K. J., Correction: Response to Comment on "Deficient NLRP3 and AIM2 Inflammasome Function in Autoimmune NZB Mice". *Journal of immunology* 2016, 197, (2), 676.

254. Vanhove, W.,Peeters, P. M.,Staelens, D.,Schraenen, A.,Van der Goten, J.,Cleynen, I.,De Schepper, S.,Van Lommel, L.,Reynaert, N. L.,Schuit, F.,Van Assche, G.,Ferrante, M.,De Hertogh, G.,Wouters, E. F.,Rutgeerts, P.,Vermeire, S.,Nys, K.. Arijs, I., Strong Upregulation of AIM2 and IFI16 Inflammasomes in the Mucosa of Patients with Active Inflammatory Bowel Disease. *Inflammatory bowel diseases* 2015, 21, (11), 2673-82.

255. Cheroutre, H.,Lambolez, F.. Mucida, D., The light and dark sides of intestinal intraepithelial lymphocytes. *Nat Rev Immunol* 2011, 11, (7), 445-56.

256. Man, S. M.,Karki, R.,Malireddi, R. K.,Neale, G.,Vogel, P.,Yamamoto, M.,Lamkanfi, M.. Kanneganti, T. D., The transcription factor IRF1 and guanylate-binding proteins target activation of the AIM2 inflammasome by Francisella infection. *Nature immunology* 2015, 16, (5), 467-75.

257. Kopfnagel, V.,Wittmann, M.. Werfel, T., Human keratinocytes express AIM2 and respond to dsDNA with IL-1beta secretion. *Experimental dermatology* 2011, 20, (12), 1027-9.

258. Vanhove, W.,Peeters, P. M.,Staelens, D.,Schraenen, A.,Van der Goten, J.,Cleynen, I.,De Schepper, S.,Van Lommel, L.,Reynaert, N. L.,Schuit, F.,Van Assche, G.,Ferrante, M.,De Hertogh, G.,Wouters, E. F.,Rutgeerts, P.,Vermeire, S.,Nys, K.. Arijs, I., Strong Upregulation of AIM2 and IFI16 Inflammasomes in the Mucosa of Patients with Active Inflammatory Bowel Disease. *Inflammatory bowel diseases* 2015.

259. Gill, S. R.,Pop, M.,Deboy, R. T.,Eckburg, P. B.,Turnbaugh, P. J.,Samuel, B. S.,Gordon, J. I.,Relman, D. A.,Fraser-Liggett, C. M.. Nelson, K. E., Metagenomic analysis of the human distal gut microbiome. *Science (New York, N.Y.)* 2006, 312, (5778), 1355-9.

260. Purchiaroni, F.,Tortora, A.,Gabrielli, M.,Bertucci, F.,Gigante, G.,Ianiro, G.,Ojetti, V.,Scarpellini, E.. Gasbarrini, A., The role of intestinal microbiota and the immune system. *European review for medical and pharmacological sciences* 2013, 17, (3), 323-33.

261. Collado, M. C.,Meriluoto, J.. Salminen, S., Role of commercial probiotic strains against human pathogen adhesion to intestinal mucus. *Letters in applied microbiology* 2007, 45, (4), 454-60.

262. Veerappan, G. R.,Betteridge, J.. Young, P. E., Probiotics for the treatment of inflammatory bowel disease. *Current gastroenterology reports* 2012, 14, (4), 324-33.

263. Dinarello, C. A., A clinical perspective of IL-1beta as the gatekeeper of inflammation. *European journal of immunology* 2011, 41, (5), 1203-17.

264. Dinarello, C. A., Immunological and inflammatory functions of the interleukin-1 family. *Annual review of immunology* 2009, 27, 519-50.

265. Herzog, C.,Haun, R. S.,Kaushal, V.,Mayeux, P. R.,Shah, S. V.. Kaushal, G. P., Meprin A and meprin alpha generate biologically functional IL-1beta from pro-IL-1beta. *Biochemical and biophysical research communications* 2009, 379, (4), 904-8.

266. Beausejour, A.,Grenier, D.,Goulet, J. P.. Deslauriers, N., Proteolytic activation of the interleukin-1beta precursor by Candida albicans. *Infection and immunity* 1998, 66, (2), 676-81.

267. Netea, M. G.,van de Veerdonk, F. L.,van der Meer, J. W.,Dinarello, C. A.. Joosten, L. A., Inflammasome-independent regulation of IL-1-family cytokines. *Annual review of immunology* 2015, 33, 49-77.

268. Kummer, J. A.,Broekhuizen, R.,Everett, H.,Agostini, L.,Kuijk, L.,Martinon, F.,van Bruggen, R.. Tschopp, J., Inflammasome components NALP 1 and 3 show distinct but separate expression profiles in human tissues suggesting a site-specific role in the inflammatory response. *The journal of histochemistry and cytochemistry : official journal of the Histochemistry Society* 2007, 55, (5), 443-52.

269. Doitsh, G.,Galloway, N. L.,Geng, X.,Yang, Z.,Monroe, K. M.,Zepeda, O.,Hunt, P. W.,Hatano, H.,Sowinski, S.,Munoz-Arias, I.. Greene, W. C., Cell death by

pyroptosis drives CD4 T-cell depletion in HIV-1 infection. *Nature* 2014, 505, (7484), 509-14.

270. Nistico, R.,Florenzano, F.,Mango, D.,Ferraina, C.,Grilli, M.,Di Prisco, S.,Nobili, A.,Saccucci, S.,D'Amelio, M.,Morbin, M.,Marchi, M.,Mercuri, N. B.,Davis, R. J.,Pittaluga, A.. Feligioni, M., Presynaptic c-Jun N-terminal Kinase 2 regulates NMDA receptor-dependent glutamate release. *Sci Rep* 2015, 5, 9035.

271. Jenkins, D.,Balsitis, M.,Gallivan, S.,Dixon, M. F.,Gilmour, H. M.,Shepherd, N. A.,Theodossi, A.. Williams, G. T., Guidelines for the initial biopsy diagnosis of suspected chronic idiopathic inflammatory bowel disease. The British Society of Gastroenterology Initiative. *Journal of clinical pathology* 1997, 50, (2), 93-105.

272. Keren, D. F.,Appelman, H. D.,Dobbins, W. O., 3rd,Wells, J. J.,Whisenant, B.,Foley, J.,Dieterle, R.. Geisinger, K., Correlation of histopathologic evidence of disease activity with the presence of immunoglobulin-containing cells in the colons of patients with inflammatory bowel disease. *Hum Pathol* 1984, 15, (8), 757-63.

273. Riley, S. A.,Mani, V.,Goodman, M. J.,Dutt, S.. Herd, M. E., Microscopic activity in ulcerative colitis: what does it mean? *Gut* 1991, 32, (2), 174-8.

274. Bryant, R. V.,Burger, D. C.,Delo, J.,Walsh, A. J.,Thomas, S.,von Herbay, A.,Buchel, O. C.,White, L.,Brain, O.,Keshav, S.,Warren, B. F.. Travis, S. P., Beyond endoscopic mucosal healing in UC: histological remission better predicts corticosteroid use and hospitalisation over 6 years of follow-up. *Gut* 2016, 65, (3), 408-14.

275. Geboes, K.,Riddell, R.,Ost, A.,Jensfelt, B.,Persson, T.. Lofberg, R., A reproducible grading scale for histological assessment of inflammation in ulcerative colitis. *Gut* 2000, 47, (3), 404-9.

276. Stahlberg, D.,Veress, B.,Mare, K.,Granqvist, S.,Agren, B.,Richter, S.. Lofberg, R., Leukocyte migration in acute colonic inflammatory bowel disease: comparison of histological assessment and Tc-99m-HMPAO labeled leukocyte scan. *The American journal of gastroenterology* 1997, 92, (2), 283-8.

277. Weldon, M. J.,Masoomi, A. M.,Britten, A. J.,Gane, J.,Finlayson, C. J.,Joseph, A. E.. Maxwell, J. D., Quantification of inflammatory bowel disease activity using technetium-99m HMPAO labelled leucocyte single photon emission computerised tomography (SPECT). *Gut* 1995, 36, (2), 243-50.

278. Cook, M. G.. Dixon, M. F., An analysis of the reliability of detection and diagnostic value of various pathological features in Crohn's disease and ulcerative colitis. *Gut* 1973, 14, (4), 255-62.

279. Vonk, A. G.,Netea, M. G.,van der Meer, J. W.. Kullberg, B. J., Host defence against disseminated Candida albicans infection and implications for antifungal immunotherapy. *Expert Opin Biol Ther* 2006, 6, (9), 891-903.

280. Kullberg, B. J.,Van'T Wout, J. W.. Van Furth, R., Role of Granulocytes in Increased Host Resistance to Candida albicans Induced by Recombinant Interleukin-1. *Infection and immunity* 1990, (58), 3319-24.

281. Joosten, L. A.,Netea, M. G.,Fantuzzi, G.,Koenders, M. I.,Helsen, M. M.,Sparrer, H.,Pham, C. T.,van der Meer, J. W.,Dinarello, C. A.. van den Berg, W. B., Inflammatory arthritis in caspase 1 gene-deficient mice: contribution of

proteinase 3 to caspase 1-independent production of bioactive interleukin-1beta. *Arthritis Rheum* 2009, 60, (12), 3651-62.

282. Cassel, S. L.,Janczy, J. R.,Bing, X.,Wilson, S. P.,Olivier, A. K.,Otero, J. E.,Iwakura, Y.,Shayakhmetov, D. M.,Bassuk, A. G.,Abu-Amer, Y.,Brogden, K. A.,Burns, T. L.,Sutterwala, F. S.. Ferguson, P. J., Inflammasome-independent IL-1beta mediates autoinflammatory disease in Pstpip2-deficient mice. *Proceedings of the National Academy of Sciences of the United States of America* 2014, 111, (3), 1072-7.

283. Lukens, J. R.,Gross, J. M.,Calabrese, C.,Iwakura, Y.,Lamkanfi, M.,Vogel, P.. Kanneganti, T. D., Critical role for inflammasome-independent IL-1beta production in osteomyelitis. *Proceedings of the National Academy of Sciences of the United States of America* 2014, 111, (3), 1066-71.

284. Coll, R. C.,Robertson, A. A.,Chae, J. J.,Higgins, S. C.,Munoz-Planillo, R.,Inserra, M. C.,Vetter, I.,Dungan, L. S.,Monks, B. G.,Stutz, A.,Croker, D. E.,Butler, M. S.,Haneklaus, M.,Sutton, C. E.,Nunez, G.,Latz, E.,Kastner, D. L.,Mills, K. H.,Masters, S. L.,Schroder, K.,Cooper, M. A.. O'Neill, L. A., A small-molecule inhibitor of the NLRP3 inflammasome for the treatment of inflammatory diseases. *Nature medicine* 2015, 21, (3), 248-55.

285. Grenier, J. M.,Wang, L.,Manji, G. A.,Huang, W. J.,Al-Garawi, A.,Kelly, R.,Carlson, A.,Merriam, S.,Lora, J. M.,Briskin, M.,DiStefano, P. S.. Bertin, J., Functional screening of five PYPAF family members identifies PYPAF5 as a novel regulator of NF-kappaB and caspase-1. *FEBS letters* 2002, 530, (1-3), 73-8.

286. Seregin, S. S.,Golovchenko, N.,Schaf, B.,Chen, J.,Eaton, K. A.. Chen, G. Y., NLRP6 function in inflammatory monocytes reduces susceptibility to

chemically induced intestinal injury. *Mucosal immunology* 2017, 10, (2), 434-445.

287. Forstner, G., Signal transduction, packaging and secretion of mucins. *Annu Rev Physiol* 1995, 57, 585-605.

288. Deplancke, B.. Gaskins, H. R., Microbial modulation of innate defense: goblet cells and the intestinal mucus layer. *The American journal of clinical nutrition* 2001, 73, (6), 1131S-1141S.

289. Birchenough, G. M.,Nystrom, E. E.,Johansson, M. E.. Hansson, G. C., A sentinel goblet cell guards the colonic crypt by triggering Nlrp6-dependent Muc2 secretion. *Science (New York, N.Y.)* 2016, 352, (6293), 1535-42.

290. Johansson, M. E., Fast renewal of the distal colonic mucus layers by the surface goblet cells as measured by in vivo labeling of mucin glycoproteins. *PloS one* 2012, 7, (7), e41009.

291. Knoop, K. A.,McDonald, K. G.,Kulkarni, D. H.. Newberry, R. D., Antibiotics promote inflammation through the translocation of native commensal colonic bacteria. *Gut* 2016, 65, (7), 1100-9.

292. Knoop, K. A.,McDonald, K. G.,McCrate, S.,McDole, J. R.. Newberry, R. D., Microbial sensing by goblet cells controls immune surveillance of luminal antigens in the colon. *Mucosal immunology* 2015, 8, (1), 198-210.

293. McDole, J. R.,Wheeler, L. W.,McDonald, K. G.,Wang, B.,Konjufca, V.,Knoop, K. A.,Newberry, R. D.. Miller, M. J., Goblet cells deliver luminal antigen to CD103+ dendritic cells in the small intestine. *Nature* 2012, 483, (7389), 345-9.

294. Jankowski, J. A.,Bedford, F. K.,Boulton, R. A.,Cruickshank, N.,Hall, C.,Elder, J.,Allan, R.,Forbes, A.,Kim, Y. S.,Wright, N. A.. Sanders, D. S., Alterations in classical cadherins associated with progression in ulcerative and Crohn's colitis. *Laboratory investigation; a journal of technical methods and pathology* 1998, 78, (9), 1155-67.

295. Gremel, G.,Wanders, A.,Cedernaes, J.,Fagerberg, L.,Hallstrom, B.,Edlund, K.,Sjostedt, E.,Uhlen, M.. Ponten, F., The human gastrointestinal tract-specific transcriptome and proteome as defined by RNA sequencing and antibody-based profiling. *Journal of gastroenterology* 2015, 50, (1), 46-57.

296. Lech, M.,Avila-Ferrufino, A.,Skuginna, V.,Susanti, H. E.. Anders, H. J., Quantitative expression of RIG-like helicase, NOD-like receptor and inflammasome-related mRNAs in humans and mice. *International immunology* 2010, 22, (9), 717-28.

297. Strugala, V.,Dettmar, P. W.. Pearson, J. P., Thickness and continuity of the adherent colonic mucus barrier in active and quiescent ulcerative colitis and Crohn's disease. *Int J Clin Pract* 2008, 62, (5), 762-9.

298. Mohanty, P.,Aljada, A.,Ghanim, H.,Hofmeyer, D.,Tripathy, D.,Syed, T.,Al-Haddad, W.,Dhindsa, S.. Dandona, P., Evidence for a potent antiinflammatory effect of rosiglitazone. *J Clin Endocrinol Metab* 2004, 89, (6), 2728-35.

299. Halbleib, J. M.. Nelson, W. J., Cadherins in development: cell adhesion, sorting, and tissue morphogenesis. *Genes Dev* 2006, 20, (23), 3199-214.

300. Schneider, M. R.,Dahlhoff, M.,Horst, D.,Hirschi, B.,Trulzsch, K.,Muller-Hocker, J.,Vogelmann, R.,Allgauer, M.,Gerhard, M.,Steininger, S.,Wolf, E..

Kolligs, F. T., A key role for E-cadherin in intestinal homeostasis and Paneth cell maturation. *PloS one* 2010, 5, (12), e14325.

301. Braga, V. M., Cell-cell adhesion and signalling. *Curr Opin Cell Biol* 2002, 14, (5), 546-56.

302. Gassler, N.,Rohr, C.,Schneider, A.,Kartenbeck, J.,Bach, A.,Obermuller, N.,Otto, H. F.. Autschbach, F., Inflammatory bowel disease is associated with changes of enterocytic junctions. *American journal of physiology. Gastrointestinal and liver physiology* 2001, 281, (1), G216-28.

303. Kucharzik, T.,Walsh, S. V.,Chen, J.,Parkos, C. A.. Nusrat, A., Neutrophil transmigration in inflammatory bowel disease is associated with differential expression of epithelial intercellular junction proteins. *The American journal of pathology* 2001, 159, (6), 2001-9.

304. Swidsinski, A.,Ladhoff, A.,Pernthaler, A.,Swidsinski, S.,Loening-Baucke, V.,Ortner, M.,Weber, J.,Hoffmann, U.,Schreiber, S.,Dietel, M.. Lochs, H., Mucosal flora in inflammatory bowel disease. *Gastroenterology* 2002, 122, (1), 44-54.

305. Kim, S.,Bauernfeind, F.,Ablasser, A.,Hartmann, G.,Fitzgerald, K. A.,Latz, E.. Hornung, V., Listeria monocytogenes is sensed by the NLRP3 and AIM2 inflammasome. *European journal of immunology* 2010, 40, (6), 1545-51.

306. Meixenberger, K.,Pache, F.,Eitel, J.,Schmeck, B.,Hippenstiel, S.,Slevogt, H.,N'Guessan, P.,Witzenrath, M.,Netea, M. G.,Chakraborty, T.,Suttorp, N.. Opitz, B., Listeria monocytogenes-infected human peripheral blood mononuclear cells produce IL-1beta, depending on listeriolysin O and NLRP3. *Journal of immunology* 2010, 184, (2), 922-30.

307. Tomalka, J.,Ganesan, S.,Azodi, E.,Patel, K.,Majmudar, P.,Hall, B. A.,Fitzgerald, K. A.. Hise, A. G., A novel role for the NLRC4 inflammasome in mucosal defenses against the fungal pathogen Candida albicans. *PLoS pathogens* 2011, 7, (12), e1002379.

308. Hise, A. G.,Tomalka, J.,Ganesan, S.,Patel, K.,Hall, B. A.,Brown, G. D.. Fitzgerald, K. A., An essential role for the NLRP3 inflammasome in host defense against the human fungal pathogen Candida albicans. *Cell host & microbe* 2009, 5, (5), 487-97.

309. Olivares-Villagomez, D.. Van Kaer, L., Intestinal Intraepithelial Lymphocytes: Sentinels of the Mucosal Barrier. *Trends in immunology* 2017.